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PRINCIPAL INVESTIGATOR: George Wilding, M.D.

CONTRACTING ORGANIZATION: University of Wisconsin-Madison
Madison, Wisconsin 53706-1490

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6. AUTHOR(S)
George Wilding, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin-Madison
Madison, Wisconsin 53706-1490

E-Mail: gxw@medicine.wisc.edu

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13. ABSTRACT (Maximum 200 Words)
The goal of this project was to identify factors that contribute to and consequences of androgen-induced oxidative stress in human prostate carcinoma cells. The JunD transcription factor was found to play a key role in androgen induced oxidative stress. Activation of oxidative stress by androgen was found to produce significant levels of cellular damage. This finding supports our hypothesis that androgen may contribute to prostate carcinogenesis through the development of oxidative stress. Antioxidant enzyme activities were significantly altered by androgen-induced stress and vitamin E treatment. Interestingly, we found that the combination of androgen and vitamin E succinate potentiated LNCaP cell death, which may have important implications for prostate cancer prevention and therapy. In our search for alternative antioxidants, we discovered that the antioxidant moiety of vitamin E is a potent inhibitor of androgenic activity. We have also discovered that oxidative stress produced by androgen exposure is not a simple manifestation of androgen receptor activation. Therefore, multiple factors contribute to androgen-induced oxidative stress and we are currently trying to identify these factors. All of the tasks performed in this project led to a much greater understanding of the mechanism and consequences of androgen to produce oxidative stress in prostate cells.

14. SUBJECT TERMS
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INTRODUCTION

Prostate cancer is the most commonly diagnosed solid tumor among men in the United States (Jemal et al., 2004). Normal prostate development and functional maintenance depend on androgens (Wilding, 1995). However, androgens are also necessary for pathological conditions that develop in the prostate gland such as prostate cancer. The importance of androgens in prostate carcinogenesis is suggested by observations that prostate cancer rarely occurs in eunuchs or men with a deficiency in 5-α reductase, the enzyme responsible for converting testosterone to its more active form, 5-α-dihydrotestosterone (DHT) (Thompson et al., 1995). These observations demonstrate that modulating androgen effects in men can prevent the development of prostate cancer. Therefore, understanding the molecular mechanisms of androgenic effects on the prostate gland could assist in the development of strategies for prostate cancer prevention.

The androgen receptor (AR) is a steroid hormone receptor that mediates the activity of androgens in androgen-responsive tissues such as the prostate gland (Wilding, 1995). Binding of androgen to the AR activates the transcription factor function of the AR resulting in altered expression of responsive genes. These genes are varied and ultimately responsible for the phenotypic characteristics associated with androgen activity. The removal of androgens by castration results in atrophy of the prostate gland. Thus, androgens act through activation of the AR, which is necessary for the normal function of the prostate gland. However, the scope of cellular changes following gene activation by the androgen receptor in the prostate gland is not well characterized.

For many years, it has been suggested that reactive oxygen species (ROS) play a role in carcinogenesis. However, only recently has evidence been provided to establish a link between oxidative stress (i.e., an increase in ROS) and tumor development in various tissues (Cerutti, 1985; Oberley and Oberley, 1993). Under certain conditions, ROS such as superoxide radicals, hydrogen peroxide and hydroxyl radicals have the ability to cause lipid peroxidation, alter the activity of thiol-dependent enzymes, or damage DNA (Dargel, 1992). DNA reacted with oxygen radicals produces more than 30 different DNA adducts (Feig et al., 1994). Recently, evidence associating oxidative damage with human prostate cancer tissue was reported (Malins et al., 1997). Alterations in glutathione S-transferase-π expression in prostate cancer also offer intriguing evidence that redox-related pathways are important in prostate cancer development (Lee et al., 1994). In addition, low levels of ROS have been demonstrated to act as mitogens, and it is proposed that redox alterations may play a key role in a novel signal transduction pathway important for regulating cell growth (Schulze-Osthoff et al., 1997).

Alterations in the redox balance of cells can also affect the activation of certain transcription factors and thus play a role in the control of gene expression (Schulze-Osthoff et al., 1997; Sen and Packer, 1996). For example, the nuclear factor κB (NF-κB) transcription factors, members of the Rel family of transcription factors, can be activated by oxidative stress (Toledano and Leonard, 1991). NF-κB proteins are located in the cytoplasm in an inactive form bound to inhibitory κB (IκB). On exposure to hydrogen peroxide or agents that increase ROS (e.g., TNF-α) I κB is degraded, releasing the NF-κB proteins and allowing them to enter the nucleus and function as transcription factors, thus modulating the expression of NF-κB responsive genes. Another group of transcription factors that respond to changes in cellular redox status include those that bind activator protein 1 (AP-1) response elements (Abate et al., 1990). Members of the Fos family of transcription factors (e.g. c-Fos, FosB, Fra-1, Fra-2) heterodimerize with Jun family members (e.g., c-Jun, JunB, and JunD), whereas Jun family members can homodimerize, to bind the AP-1 response element. AP-1 binding factors are further regulated in their activity by phosphorylation. Therefore, changes in cellular redox status can

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have pronounced effects on transcription factor activity, resulting in significant changes in cellular gene expression.

Removal of the different ROS is accomplished by specific antioxidants and antioxidant enzymes, which regulate cellular redox status. For example, glutathione (GSH) provides protection from superoxide and hydroxyl radicals, and singlet oxygen, while vitamin E (i.e., α tocopherol) primarily reacts with lipid alkoxys or peroxyl radicals (Halliwell and Gutteridge, 1999). The superoxide dismutases reduce superoxide radicals into hydrogen peroxide, which is further reduced by glutathione peroxidase or catalase (Michiels and Remacle, 1988; Oberley and Oberley, 1986). Other antioxidant enzymes that have been shown to modulate transcription factor activity include thioredoxin peroxidase (Jin et al., 1997). The subcellular localization of these antioxidants and antioxidant enzymes, in relation to the site of reactive oxygen production, is a further determinant of whether a given ROS will be reduced.

The mechanisms controlling the prooxidant-antioxidant balance of cells are not well understood. In this project, we investigated a role for androgens in the regulation of the cellular redox state in human prostate cells. The ability of androgens to modulate oxidative stress, either through the production of ROS or through alteration of the antioxidant defense capabilities, could potentially have an effect on growth and detoxification processes. In the current project, we hypothesized that *androgen exposure in the prostate gland leads to the development of ROS, which contributes to the development of prostate cancer.* Three aims were proposed to: 1) examine the role of redox-sensitive transcriptional pathways in androgen-induced oxidative stress in LNCaP prostate cells; 2) to evaluate the role of antioxidant enzyme pathways in LNCaP cells, and to; 3) further validate the mechanisms of androgen receptor activation in androgen-induced oxidative stress in human prostate cells. This report summarizes the progress that was achieved from the original Statement of Work over the course of this project.

REPORT BODY

A list of acronyms and abbreviations used in this report is included as Appendix 1. All data, figures, and tables referred to in this report are included as part of the appendix. Where appropriate, reference is made to data presented in either the first or second Annual Report. However, some of this information has been modified as requested in the last Annual Report and is included in this Final Report. In addition, some of the information included in this report has been published or has been prepared for publication and these manuscripts have been appended to this report. Reference to the relevant data in these manuscripts is included in the Report Body. Every attempt has been made to make sure that this final report is comprehensive; therefore, data from previous reports has been reiterated to stress the important findings of this project.

The NF-κB and AP-1 transcription factors are susceptible to changes in cellular redox status. The aim of Task 1 was to determine the role of the redox-sensitive NF-κB and AP-1 transcription factor activation in LNCaP cells after androgen exposure. We have previously shown that binding of the NF-κB and AP-1 transcription factors to their response elements are increased in LNCaP cells after exposure to androgen (Ripple et al., 1999). The first study (Task 1a) was performed to assess the changes in IκBα and IκBβ levels, which are responsible for regulating NF-κB activity, in LNCaP cells after exposure to the androgen R1881 for 4 days. Immunoblot analyses were performed using antibodies specific to either IκBα or IκBβ (Appendix 2, Figure 1). No change in IκBβ levels were observed after exposure to either 0.1 or 1.0 nM R1881 for 4 days in LNCaP cells (Appendix 2, Figure 1c). However, a significant 20% decrease in IκBα levels was found after exposure to 1 nM R1881, whereas no change in

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IκBα levels was found after exposure to 0.1 nM R1881 for 4 days (Appendix 2, Figure 1a&b). Therefore, exposure to a growth inhibitory dose of 1nM R1881 for 4 days in LNCaP cells was found to decrease cellular IκBα levels, which is consistent with the activation of NF-κB found in LNCaP cells after androgen exposure.

NF-κB transcription factors are found in an inactive state localized to the cytoplasm due to binding by IκB (Huang et al., 2000). Stimuli, such as oxidative stress, that leads to IκB degradation results in the release of NF-κB transcription factors. To further assess the role of NF-κB in androgen-induced oxidative stress in LNCaP cells, we proposed to develop LNCaP cell lines that are stably transfected with plasmid vectors that express either wild-type IκBα as a control vector or a super-suppressor form of IκBα that inactivates NF-κB expression by irreversibly binding NF-κB (Task 1b). Dr. Shigeki Miyamoto (U.W. Department of Pharmacology) provided the vectors used and was consulted regarding the experimental design for in these studies. The super-suppressor form of IκBα is a mutant form of IκBα that is not ubiquitinated and therefore is resistant to proteasomal degradation. In contrast, expression of wild-type IκBα is susceptible to proteasomal degradation and thus serves as the most suitable control for altered expression levels of IκBα produced by expression vector transduction. Polyclonal LNCaP lines were produced that express either wild-type IκBα or mutant IκBα and characterized for growth response and reactive oxygen species (ROS) production following androgen exposure (Appendix 2, Figure 2 a&b). Neither the expression of mutant nor wild-type IκBα affected cellular response to androgen in these lines (Appendix 2, Figure 2). For example, in LNCaP cells expressing mutant IκBα, which would be expected to inhibit androgen-induced NFκB translocation, the androgen-mediated growth curve was not significantly affected compared to control LNCaP cells expressing wild-type IκBα or untransduced LNCaP cells (Appendix 2, Figure 2a). Also, ROS production, as measured using the DCF assay, in LNCaP cells transduced with mutant and wild-type IκBα compared to control, untransduced LNCaP cells, was not affected (Appendix 2, Figure 2b). Additionally, six stably transduced clones expressing wild-type IκBα and six clones expressing mutant IκBα were produced and examined for growth and ROS production after androgen exposure. As observed with the polyclonal mutant and wild-type IκBα lines, the clonal IκBα lines did not produce an altered growth or ROS response after androgen exposure (results similar to those presented in Appendix 2, Figures 2a & 2b). In response to concerns stated in the review of the Second Annual Report (i.e., Technical Issues), studies were performed to determine changes in LNCaP response to androgen using cells harboring a backbone control vector. The androgenic response of LNCaP cells harboring a backbone control vector was comparable to untransduced LNCaP cells, further validating our reported results. Results with the mutant and wild-type IκBα lines suggest that NF-κB activity is not necessary for androgen-induced oxidative stress in LNCaP human prostate carcinoma cells. This observation is in agreement with our current hypothesis on the role of NF-κB in androgen-induced oxidative stress in LNCaP cells (see Conclusions).

As part of Task 1, we also investigated the role of AP-1 transcription factors in oxidative stress produced after androgen exposure in LNCaP cells. Our initial studies to characterize AP-1 factors induced by androgen exposure are in press and appended to this report (Appendix 3). This report showed that alterations in AP-1 factors produced by androgen exposure could lead to changes in AP-1-mediated transcriptional activity (Appendix 3). Many of the effects on AP-1 factors produced by androgen exposure in LNCaP cells, as presented in the appended manuscript (Appendix 3), were blocked by administration of the antiandrogen bicalutamide, suggesting a central role for the AR in androgen-induced oxidative stress in LNCaP cells. This observation is of key importance for the studies proposed in Task 3. Importantly, we found that the JunD AP-1

factor was increased by androgen exposure in LNCaP cells through an AR-mediated pathway (Appendix 3).

To determine whether or not JunD was sufficient to produce changes in LNCaP growth and redox changes, a vector expressing JunD and co-expressing enhanced green fluorescence protein (EGFP) was transfected into LNCaP cells and positive and negative cells were identified and isolated by flow cytometry. The EGFP vector used in this study, pIRES2-EGFP (BD Biosciences) was found to be useful for many of the studies in this project. The structure of the backbone construct is shown in Appendix 2, Figure 3. Vector control studies were performed using the EGFP expressing backbone plasmid, pIRES2.EGFP. At 48 hours, no significant changes in LNCaP growth or ROS production were observed (Appendix 2, Figure 4a&b). However, after 96 hours, a significant decrease in LNCaP growth and a significant increase in ROS production were observed in LNCaP cells following transduction of a JunD expressing vector (Appendix 2, Figure 4a&b). The kinetics of JunD-induced growth effects and ROS production are similar to those produced by androgen-exposure in LNCaP cells. Therefore, JunD expression is sufficient to produce a decrease in the rate of LNCaP growth and an increase in LNCaP cellular ROS production, similar to that produced by androgen exposure.

To assess the necessity of JunD in androgen-induced oxidative stress in LNCaP cells, LNCaP lines that stably express a dominant-negative form of JunD were developed. A schematic of the constructs used is shown in Appendix 2, Figure 5a. pIRES2-EGFP was used as the backbone control for these experiments. The dominant-negative form of JunD from human *jun*D cDNA was constructed and has been designated JunDΔTA. JunDΔTA was subcloned into pIRES2-EGFP and was used to determine the effects of dominant-negative JunD activity on androgen-induced AP-1 activation (Appendix 2, Figure 6). JunDΔTA expression was found to significantly inhibit androgen-induced AP-1 activation in LNCaP cells at both 24 and 96 hours (Appendix 2, Figure 6). Thus, a vector that expresses a dominant-negative form of JunD protein that can inhibit androgen-induced AP-1 activation was developed.

Clonal cell lines that stably express the backbone pIRES2-EGFP and JunDΔTA expression vector were produced to assess the action of dominant-negative JunD (i.e., JunDΔTA) activity on androgen-induced growth changes and oxidative stress in LNCaP cells (Appendix 2, Figure 5b). Five control pIRES2-EGFP clones were characterized for JunD expression, growth response to 1 nM R1881, and production of ROS after exposure to 1 nM R1881. Immunoblot analysis of nuclear extracts demonstrated that the JunD protein expression profile of the control clones was comparable to the parental LNCaP cell line and the response of control clones to 1 nM R1881 was comparable to the parental LNCaP line (Appendix 2, Figure 5b). Therefore, expression of the pIRES2-EGFP backbone did not affect LNCaP JunD protein or response to androgen. Immunoblot characterization of nuclear extracts from the JunDΔTA clones 1, 3, 4, and 5 showed the expression of a unique band at M_r 29,000 that corresponds to JunDΔTA, whereas JunDΔTA clone 2 did not demonstrably express the JunDΔTA protein band (Appendix 2, Figure 5b). Importantly, in clones expressing JunDΔTA, the growth and ROS responses after exposure to 1 nM R1881 for 4 days were inhibited (Appendix 2, Figure 7a&b). Thus, the combined results of Task 1c suggest that JunD is both necessary and sufficient for the production of androgen-induced oxidative stress in LNCaP cells.

The aim of Task 2 of this project was to determine changes in the levels and activity of antioxidant enzymes and oxidative damage products in response to androgen-induced oxidative stress in LNCaP cells and to determine what effect vitamin E exposure has on these parameters. Changes in the expression levels of antioxidant enzyme transcripts were assessed by microarray analysis of LNCaP cells treated with control medium or medium containing 1 nM R1881 for 4 days (Appendix 2, Table 1). Interestingly, the mRNA expression of most antioxidant enzymes

examined, including catalase (CAT), copper-zinc superoxide dismutase (Cu/ZnSOD, manganese superoxide dismutase (MnSOD), glutathione peroxidases (GPxs), peroxiredoxins and thioredoxin were either not significantly altered or only very modestly changed (i.e., 1.5- to 1.8-fold). In contrast, heme oxygenase mRNA, which is not considered an antioxidant enzyme but may be a marker of oxidative stress, was found to be very significantly increased 32.3-fold (Appendix 2, Table 1). Additionally, thioredoxin reductase 1 levels were increased 2.8-fold, which may be significant as the absolute expression levels rose from a value of 1954 to 5540 (Appendix 2, Table 1). Thus, changes in mRNA levels of antioxidant enzymes may not serve as good markers of androgen-induced oxidative stress.

Antioxidant enzyme profiles were also determined to assess the action of androgen-induced oxidative stress in LNCaP cells. Protein levels of the antioxidant enzymes MnSOD, CuZnSOD, and CAT were determined in LNCaP human prostate carcinoma cell lines following exposure to androgen and vitamin E at 24 and 96 hours (Appendix 2, Figure 8). Although no significant changes in antioxidant enzyme protein levels were observed, significant changes in MnSOD, CuZnSOD, CAT, and GPx activity were produced by androgen and vitamin E treatment (Appendix 2, Tables 2&3). For example, at 24 hours, CAT activity was increased 1.4-fold in LNCaP cells treated with 20 µM vitamin E succinate and decreased 44% in LNCaP cells co-administered 1 nM R1881 and 20 µM vitamin E succinate (Appendix 2, Table 2). Importantly, all antioxidant enzyme activities assessed at 96 hours were significantly affected by 1 nM R1881 and vitamin E co-administration (Appendix 2, Table 3). For example, at 96 hours, 0.05 nM R1881, with or without vitamin E, produced a 14% decrease in CuZnSOD activity and the co-administration of 1 nM R1881 and 20 µM vitamin E decreased CuZnSOD activity 20% (Appendix 2, Table 3). In constrast, MnSOD activity was decreased approximately 60% by 20 µM vitamin E succinate treatment and increased 1.6- and 1.9-fold by exposure to 1 nM R1881 and the combination of 1 nM R1881 and 20 µM vitamin E succinate, respectively. CAT activity was decreased approximately 90% by treatment with 20 µM vitamin E, with or without 1 nM R1881, and GPx activity was decreased 80% by exposure to 1 nM R1881 and 20 µM vitamin E for 96 hours (Appendix 2, Table 3). Thus, both androgen exposure and vitamin E treatment significantly affected the activity of antioxidant enzymes in LNCaP human prostate carcinoma cells, which was not accurately reflected by changes in antioxidant enzyme mRNA or protein levels in these cells.

Recent studies have demonstrated that peroxiredoxin proteins play a critical role in cellular redox. Therefore, protein levels of peroxiredoxin I, II, III, IV, V, and VI were determined after treatment with 0.05 or1 nM R1881 and/or 20 µM vitamin E succinate for 4 days (Appendix 2, Figures 9a, b, & c). No changes were observed in the protein levels of peroxiredoxin I, II, and VI after androgen or vitamin E exposure (Appendix 2, Figure 9a). A small decrease in peroxiredoxin III levels was observed after exposure to the combination of androgen and vitamin E. For peroxiredoxin IV and V, protein levels of the primary band were not altered by exposure to androgen or vitamin E. However, a higher MW band became prominent in cells exposed to 1 nM R1881 for 4 days with or without vitamin E (Appendix 2, Figure 9a & 9b). Thus, post-translation events may selectively alter the peroxiredoxin expression in LNCaP cells exposed to androgen and vitamin E.

The last part of Task 2 was to determine the effects of androgen-induced oxidative stress on the production of oxidative damage products and how these products could be modulated by vitamin E succinate treatment. We found that treatment of LNCaP cells with either 1 nM R1881 or 20 µM vitamin E succinate alone did not affect LNCaP viability (Appendix 2, Figure 10a,b&c). However, the co-administration of 1 nM R1881 and 20 µM vitamin E succinate significantly decreased LNCaP viability (Appendix 2, Figure 10d&e). This was demonstrated by

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numerous floating cells in cultures that were co-administered R1881 and vitamin E succinate (Appendix 2, Figure 10d), the generation of a hypodiploid population of cells (Appendix 2, Figure 11 a-d), and significant cellular damage that could be observed by electron microscopy (Appendix 2, Figure 12). Treatment of LNCaP cells with 1 nM R1881 or 20 µM vitamin E succinate to LNCaP cells was found to produce increased lipid hydroperoxide levels compared to control untreated cells (Appendix 2, Figure 13a). The highest lipid hydroperoxide levels were observed in cells co-administered 1 nM R1881 and 20 µM vitamin E succinate (Appendix 2, Figure 13a). Increased levels of 4-hydroxy-2-nonenal (4-HNE) protein adducts were observed in LNCaP cells treated with 1 nM R1881 or the combination of 1 nM R1881 and 20 µM vitamin E succinate (Appendix 2, Figure 13b, 14, and Table 4). Malondialdehyde (MDA) levels were not found to be significantly changed in cells exposed to 1 nM R1881 (data not shown). Interestingly, the GSH/GSSG ratio, a sensitive indicator of cellular redox state, was increased at 24 hours in LNCaP cells treated with 20 µM vitamin E succinate, but unchanged at 96 hours (Appendix 2, Figure 15). In contrast, the administration of 1 nM R1881, either with or without 20 µM vitamin E succinate, resulted in a significant decrease in the GSH/GSSG ratio at 96 hours, suggesting the presence of significant cellular oxidative stress in androgen exposed LNCaP cells, which was not significantly altered by vitamin E succinate treatment (Appendix 2, Figure 15). Thus, the presence of oxidative damage products after androgen and vitamin E treatment was dependent on the oxidative end product under examination.

The goal of Task 3 of this project was to determine the levels and variability of androgen-induced oxidative stress in human prostate epithelial cells (HPECs), before and after vitamin E exposure, as mediated by AR expression and activation. We established immortalized and primary cultures of HPECs with the assistance of personnel from Dr. David Jarrard's laboratory (U.W. Dept. of Surgery). An initial characterization of HPEC's was performed to determine their response to androgen exposure. LNCaP cells and HPECs were exposed to R1881 ranging in concentration from 0.001 to 10 nM. The growth of androgen-sensitive LNCaP cells was significantly altered by androgen exposure (Appendix 2, Figure 16). However, HPECs did not respond to androgen exposure (Appendix 2, Figure 16), suggesting these cells do not express the AR. Further evaluation by immunoblot analysis showed that the HPECs used in these studies did not express AR protein (data not shown). The absence of AR expression in HPECs was necessary for the studies proposed in Task 3, because the effects of androgen exposure will be determined after the AR is introduced into HPECs. DNA transduction procedures were developed to enable gene expression in a wide range of prostatic cancer and epithelial cell lines, including DU145, LNCaP, PC3 human prostate cancer cell lines and immortalized HPECs. The efficiency of 5 different chemically based transfection methods (i.e., Superfectin and Effectene [Qiagen], Lipofectamine and Lipofectamine 2000 [Gibco], and Fugene 6 [Roche]) were evaluated (Appendix 2, Figure 17). The Fugene 6 transfection method was found to be the most effective chemically based gene transduction method for DU145 cells, whereas the Superfectin method was most effective in LNCaP cells (Appendix 2, Figure 17). Similarly, the Fugene 6 transfection method was found to be best for PC3 cell transfections (data not shown). Chemically based gene transduction methods did not produce acceptable levels of DNA transduction in primary cultures of HPECs. An AR expression vector was constructed to produce AR expression in HPECs (Appendix 2, Figure 18a). To enable the identification of cells harboring the AR, the human AR was subcloned from the phCMVAR vector (kindly provided by Dr. Wayne Tilley) and placed in the multiple cloning site of the pIRES2-EGFP vector, which can be analyzed for EFGP expression using flow cytometry. This vector was designated pGFPAR (Appendix 2, Figure 18a).

To determine whether or not pGFPAR could be analyzed for green fluorescence protein

expression, PC3 cells transfected with pGFPAR using Fugene 6 were analyzed by flow cytometry (Appendix 2, Figure 18 b&c). PC3 transduced with the pGFPAR were found to be up to 86% positive for GFP expression (Appendix 2, Figure 18c). To determine the functionality of the AR expressed from pGFPAR, PC3 human prostate carcinoma cells, which do not express an AR and are not responsive to androgen, were cotransfected with a plasmid expressing luciferase driven by the mouse mammary tumor virus (MMTV) promoter (pMMTV-luciferase), which is activated by the AR. Additionally, control experiments were performed using PC3 cells cotransfected with the pIRES2-EGFP backbone vector and pMMTV-luciferase. Twenty-four hours after transfection, cells were exposed to 1 nM R1881 and luciferase expression levels were measured after 48 hours. No difference in luciferase expression levels was observed in PC3 cells cotransfected with the pIRES2-EGFP and pMMTV-luciferase vectors after R1881 treatment (Appendix 2, Figure 19a). However, PC3 cells cotransfected with pGFPAR and the pMMTV-luciferase showed a 2.5-fold increase in luciferase expression after 48 hours of 1 nM R1881 treatment (Appendix 2, Figure 19a). Thus, the AR expressed from the pGFPAR vector was found to produce androgen-dependent transcriptional activation. Further studies were performed to examine the effects of vitamin E succinate treatment on promoter activation by the AR in PC3 cells. Vitamin E succinate treatment did not affect androgen activation of the androgen-responsive MMTV promoter (Appendix 2, Figure 19b). Therefore, vitamin E succinate treatment should not interfere with studies assessing the effects of AR on oxidative stress by altering pGFPAR expression.

Our final goal in Task 3 was to determine the effects of exogenously introduced AR on the production of oxidative stress in prostate cells. To initiate these studies, the production of oxidative stress, as measured using the DCF assay, was determined in PC3 cells transduced with pGFPAR. pIRES2-EGFP was used as a backbone vector control in these studies. To date, we have not observed ROS production in PC-3 cells transduced with pGFPAR, with or without androgen treatment. Similar experiments were also performed using DU145 and immortalized HPECs, again without measurable ROS production (data not shown). Because we have demonstrated that the AR produced by pGFPAR is functional, the simplest explanation for this result is that AR activation does not generally lead to ROS production. However, we are continuing our studies to determine if AR expression and activation results in ROS production in HPECs.

Because chemically based gene transduction methods were not effective in HPECs an adenovirally mediated gene transduction system was developed. Using a LacZ expressing adenoviral vector with a multiplicity of infection of 100 per cell, nearly complete gene transfer was achieved in immortalized HPECs and primary human prostate epithelial cells with little or no toxicity (Appendix 2, Figure 20). Adenoviral vectors expressing the human AR are currently being constructed. Once constructed, adenoviral vectors will be produced to transduce the AR into HPECs and parameters of oxidative stress will be examined. A current challenge is to develop an adenoviral expression vector that will allow the simultaneous expression of the AR along with a screenable marker, such as green fluorescence protein. However, because of the high infection efficiency of HPECs achieved with adenoviral vectors, androgen induced oxidative stress may be measurable without the need for cell selection. Although these studies were not completed before the term of funding, they are close to completion and will be continued.

KEY RESEARCH ACCOMPLISHMENTS

- Determined levels of IκBα and IκBβ in LNCaP cells after androgen exposure in LNCaP cells. Found that IκBα, but not IκBβ, protein levels were decreased by androgen exposure.

- Cell lines expressing wild-type and mutant IκBα were evaluated for androgenic modulation of growth and ROS production. No change in androgen-mediated cell growth or ROS production was found in LNCaP clones expressing a super-suppressor IκBα.

- The Fra-2 and JunD AP-1 transcription factors were found to be differentially modulated by androgen exposure in LNCaP cells, which was shown to be dependent on androgen receptor activation.

- Produced wild-type JunD expression vector. Using this vector, we found that JunD decreased cell growth and produced ROS in LNCaP cells, similar to that observed with androgen exposure.

- Produced a dominant-negative JunD expression vector. Expression of dominant-negative JunD was found to inhibit androgen-induced AP-1 activation.

- Cell lines expressing dominant-negative JunD were produced and characterized for dominant-negative JunD protein expression. Cells expressing dominant-negative JunD did not exhibit growth inhibition after androgen exposure and did not produce ROS after androgen exposure.

- Determined how ROS production in LNCaP cells were changed after androgen exposure was modulated by vitamin E succinate treatment..

- Determined mRNA levels of antioxidant enzymes in LNCaP cells exposed to androgen.

- Measured protein levels of catalase, CuZnSOD, MnSOD, and peroxiredoxins (I-VI) in LNCaP cells exposed to androgen and vitamin E succinate.

- Established that MnSOD, CuZnSOD, catalase, and glutathione peroxidase activity was significantly altered in human prostate carcinoma cell lines exposed to androgen and vitamin E for 24 and 96 hours.

- Determined changes in reduced and oxidized glutathione levels in LNCaP prostate cells exposed to androgen and vitamin E.

- Performed viability analysis of LNCaP cells exposed to androgen and vitamin E using trypan blue exclusion, multiparameter flow cytometry, and light microscopy

- Used electron microscopy to evaluate changes in LNCaP cells after androgen and vitamin E treatment. Determined levels of 4-HNE produced in these cells.

- Evaluated the levels of lipid hydroperoxide produced in LNCaP cells exposed to androgen and vitamin E succinate.

- Determined that malondialdehyde levels were not altered in LNCaP cells exposed to androgen and vitamin E succinate.

- Discovered that the antioxidant moiety of vitamin E, pentamethylchromanol, potently inhibits the activity of the androgen receptor.

- Established cultures of immortalized and primary human prostate epithelial cells and determined that these cells do not express the androgen receptor and are not responsive to androgen exposure.

- Chemical-based transfection procedures were evaluated and optimized for expression vector transduction of prostate cell lines and primary prostate cells.

- Used adenoviral vectors to transduce human prostate epithelial cells in culture with high efficiency.

- Produced a screenable androgen receptor expression vector. Found vector to functionally express androgen receptor in PC3 human prostate carcinoma cells.

- Found that vitamin E succinate does not interfere with androgen receptor activation of an androgen-responsive promoter in PC3 cells.

REPORTABLE OUTCOMES

- Thompson TA and Wilding G. Androgen antagonist activity by the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol in human prostate carcinoma cells. Molec Cancer Therapeutics 2:797-803, 2003 (Appendix 3)

- Church DR, Lee E, Thompson TA, Basu HS, Ripple MO, Ariazi EA, Wilding G. Induction of AP-1 activity by androgen activation of the androgen receptor in LNCaP human prostate carcinoma cells. The Prostate (published online in Wiley InterScience, December 2004; Appendix 4)

- Zhang Y, Thompson TA, Rasoul JA, Church DR, Zhong W, Wilding G, Oberley TD. Vitamin E succinate modulates androgen-induced oxidative stress in LNCaP human prostate carcinoma cells. (Manuscript in preparation)

- Zhang Y, Thompson TA, Church DR, Santos M, Zhong W, Wilding G, Oberley TD Vitamin E succinate potentiates apoptosis in the presence of androgens in LNCaP human prostate carcinoma cells. (Manuscript in preparation)

- Thompson TA, Wilding G. Differential effects on androgenic responses in prostate cells

by the phenolic antioxidants butylated hydroxyanisole and butylated hydroxytoluene. (Manuscript in preparation)
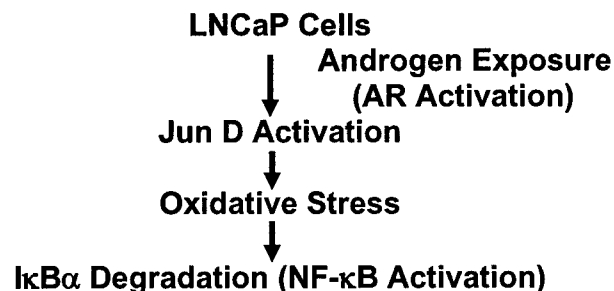
- Thompson TA, Church DR, Basu HS, Zhong W, Zhang Y, Oberley TD, Wilding G. Androgen induction of terminal differentiation in LNCaP human prostate carcinoma cells. (Manuscript in preparation)

- Lee E, Church DR, Thompson TA, Wilding G. Necessity of JunD expression and activity for regulation of growth and redox status in androgen-responsive prostate carcinoma cells. (Manuscript in preparation)

- Lee E, Thompson TA, Basu HS, Church DR, Wilding G. Review: oxidative stress and prostate cancer. (Manuscript in preparation)

- Thompson TA, Wilding G. Inhibition of androgen-mediated responses in human prostate carcinoma cells by the antioxidant moiety of vitamin E, pentamethylchromanol. (Abstract submitted for AACR 94th Annual Meeting 2003)

- Zhang Y, Thompson TA, Church D, Lucido M, Zhong W, Wilding G, Oberley TD. Vitamin E modulates androgen induced oxidative stress and potentiates cell death in LNCaP human prostate carcinoma cells. (Poster presented at Society for Free Radical Biology and Medicine 10th Annual Meeting 2003)

- Thompson TA, Church DR, Zhong W, Oberley TD, Wilding G. Terminal differentiation induction in LNCaP human prostate cancer cells. (Poster presented at AACR 95th Annual Meeting 2004)

- Lee E and Wilding G. JunD expression and activity in LNCaP prostate carcinoma cells. (Poster presented at the American Association of Cancer Research Pathobiology Workshop, July 2004)

- Lee E. The role of JunD in androgen-induced changes in prostate gland redox status. (Presented at O'Brien Center/Prostate Research Group Seminar, University of Wisconsin-Madison, September 2004)

- Lee E, Church DR, Amlong CA, Thompson TA, Wilding G. Role of JunD in androgen-induced changes in prostate gland redox status. (Poster presented at the O'Brien Urology Research Center Fall Forum, University of Wisconsin-Madison, November 2004.)

- Thompson TA and Wilding G. Method of modulating androgen-mediated responses using chroman-derived anti-androgens. US Provisional Patent Application No. 60/450,510; Utility Patent Application No. 10/789,835 (Filed 02-27-2004) (Pending)

- Plasmids developed: JunD+EGFP (vector co-expressing JunD and enhanced green fluorescent protein), JunDΔTA+EGFP (vector co-expressing dominant negative JunDΔTA, a JunD mutant created from human *jun*D cDNA, and enhanced green fluorescent protein), pGFPAR (vector co-expressing Androgen Receptor and enhanced green fluorescent protein)

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- Cell lines developed: polyclonal and clonal LNCaP – wtIκBα cell lines (wild type IκB suppressor lines), polyclonal and clonal LNCaP – mIκBα cell lines (mutant IκB suppressor lines); clonal LN/GFP and LN/JDΔTA cell lines (vector control and JunD suppressor LNCaP cell lines); polyclonal and clonal PC3GFP and PC3AR cell lines (vector control and androgen receptor expressing PC-3 cell lines)

CONCLUSION

The results of this project support our original hypothesis. That is, that *androgen exposure in prostate cells alters cellular redox, which may contribute to the development of prostate cancer.* The first task of this project was to investigate the role of redox-sensitive transcription factors in androgen-induced oxidative stress. IκBα levels were decreased in LNCaP cells by doses of androgen that induce oxidative stress. Thus, IκBα levels may serve as a useful marker of oxidative stress in prostate cells. However, inhibition of NFκB translocation using a super-suppressor IκBα did not affect androgen-induced ROS production in LNCaP cells. Therefore, NF-κB itself may not be necessary for androgen-induced oxidative stress in LNCaP cells. We hypothesize that changes in cellular redox that result from androgen exposure may play a role in NF-κB activation. For example, we have previously reported that antioxidant treatment can reduce androgen-induced NF-κB activation (Ripple et al., 1999). Thus, androgen-induced oxidative changes may contribute to NF-κB activation after androgen exposure.

Interestingly, the redox-sensitive JunD transcription factor was found to recapitulate growth effects and ROS production as observed following androgen exposure in LNCaP cells. Expression of a dominant-negative JunD was found to inhibit androgen-induced AP-1 activation in LNCaP cells. These studies clearly demonstrate a role for AP-1 in androgen-induced oxidative stress. Thus, we have found that select members of the AP-1 family of transcription factors may have a central role in androgen-induced oxidative stress, whereas other redox-sensitive transcription factors, such as NFκB, may be activated as a consequence of androgen-induced oxidative stress. The following pathway is proposed to illustrate the sequence of events in activation of redox-sensitive transcription factors based on the results of the studies performed in this project:

LNCaP Cells

↓ **Androgen Exposure**
(AR Activation)

Jun D Activation

↓

Oxidative Stress

↓

IκBα Degradation (NF-κB Activation)

The aim of Task 2 of this project was to determine if androgen exposure in LNCaP cells produced demonstrable markers of oxidative stress and whether or not vitamin E treatment could modulate endpoints of androgen induced oxidative stress. Markers of cellular damage were observed by light microscopic and electron microscopy analysis. Interestingly, LNCaP viability was not affected by high-level androgen (e.g., up to 10 nM R1881) or vitamin E succinate treatment up to 20 μM. Yet, the combination of androgen and vitamin E succinate resulted in cell death, demonstrated by floating cells, an increase in the hypodiploid population of cells, and

decreased viability, as measured by trypan blue exclusion. Additionally, oxidative damage, as measured by changes in GSH/GSSG ratio, lipid hydroperoxides, and 4-HNE adducts were increased following androgen and vitamin E exposure in LNCaP cells. We believe the increase in lipid hydroperoxides with androgen or vitamin E succinate alone may be independent cellular insults, which are additive, but not synergistic, after treatment with the combination of androgen and vitamin E succinate. We are currently performing further electron microscopic studies of LNCaP cells exposed to androgen, vitamin E, and the combination, paying particular attention to lysosomal and mitochondrial organelles, which we believe may be critical participants in the oxidative stress produced by androgen and vitamin E exposure.

The production of cellular oxidative damage did not translate into changes in antioxidant enzyme levels. For example, results assessing changes in antioxidant enzymes and oxidative damage produced by androgen-induced oxidative stress were highly varied, dependent on the specific system under evaluation. For example, transcript levels of antioxidant proteins such as catalase, Cu/ZnSOD, MnSOD, glutathione peroxidases, and peroxiredoxins were largely unchanged by androgen exposure in LNCaP cells. Similarly, the protein levels of these enzymes were largely unaffected by androgen-induced oxidative stress in LNCaP cells. However, large changes in enzyme activity were found after exposure to androgen and/or vitamin E exposure in LNCaP cells. We believe this finding is of great significance. The results of this study suggest that the best indicators of oxidative stress in cells were reflected by the activity of antioxidant enzymes, and not in their protein levels. This may have implications on the biological role of these systems as cellular damage defense mechanisms. The activity of antioxidant systems may require their immediate action in response to oxidative stress and, thus, changes in antioxidant enzyme protein levels may not be the most appropriate markers of their biological activity. We have recently observed that many of the pathways associated with changes in cellular redox after androgen exposure in LNCaP cells are coincident with the development of a more differentiated phenotype in these cells (e.g., changes in GSH/GSSG ratio). This data was presented at the 2004 Annual American Association of Cancer Research meeting.

Finally, so what? All of the tasks performed in this project led to a much greater understanding of the role of androgen to produce oxidative stress in prostate cells. For example, we found that the JunD transcription factor plays a key role in androgen induced oxidative stress in prostate cells and that NF-κB activation is a response to androgen-induced oxidative stress. Thus, we believe the AP-1 pathway may serve as a useful target to develop modalities for modulating androgen-induced oxidative stress, possibly to develop strategies for prostate cancer prevention. NF-κB activation may serve as a useful marker in evaluating cellular stress responses. Activation of oxidative stress by androgen in prostate cells was found to produce significant levels of cellular damage. This finding supports our hypothesis that androgen may contribute to prostate carcinogenesis through the development of oxidative stress. However, we did not find that vitamin E succinate was useful in curtailing cellular oxidative damage. In fact, we found that the combination of androgen and vitamin E succinate potentiated LNCaP cell death, which may have important implications for prostate cancer therapy. In addition, we have found that the many different forms of vitamin E have highly variable cellular activities and that the actions of vitamin E succinate may be distinct from other forms of vitamin E. We are currently trying to identify alternative antioxidants that are useful in reducing oxidative damage produced by androgen exposure. In our search for alternative antioxidants, we discovered that the antioxidant moiety of vitamin E, pentamethylchromanol, is, in fact, a potent inhibitor of androgenic activity (see attached manuscript). This was a completely novel observation, which challenges the traditional concept of vitamin E to act solely as an antioxidant. We have also discovered that oxidative stress produced by androgen exposure is not a simple manifestation of

AR activation. Therefore, multiple factors contribute to androgen-induced oxidative stress and we are currently trying to identify these factors. Interestingly, we have found that androgen-induced oxidative stress is coincident with a terminally differentiated phenotype in LNCaP cells, which, again, may have important implications for developing new modalities of prostate cancer prevention and therapy. Very significant findings toward our understanding of androgen-induced oxidative stress were achieved as a result of this study, which continue to support our hypothesis for a role of androgen in prostate carcinogenesis through changes in cellular redox.

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Journal and meeting abstract publications resulting from this research effort include:

Thompson TA and Wilding G. Androgen antagonist activity by the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol in human prostate carcinoma cells. Molecular Cancer Therapeutics 2:797-803, 2003.

Church DR, Lee E, Thompson TA, Basu HS, Ripple MO, Ariazi EA, Wilding G. Induction of AP-1 activity by androgen activation of the androgen receptor in LNCaP human prostate carcinoma cells. The Prostate (published online in Wiley InterScience, December 2004; in press)

Thompson TA, Wilding G. Inhibition of androgen-mediated responses in human prostate carcinoma cells by the antioxidant moiety of vitamin E, pentamethylchromanol. Proceedings of the American Association for Cancer Research, Volume 44, March 2003, p. 818, Abstract 3578 (poster session)

Zhang Y, Thompson TA, Church D, Lucido M, Zhong W, Wilding G, Oberley TD. Vitamin E modulates androgen induced oxidative stress and potentiates cell death in LNCaP human prostate carcinoma cells. Free Radical Biology and Medicine 2003; 35 Supplement 1. (poster, 10th Annual Meeting SFRBM)

Thompson TA, Church DR, Zhong W, Oberley TD, Wilding G. Terminal differentiation induction in LNCaP human prostate cancer cells. Proceedings of the American Association for Cancer Research, Volume 45, March 2004, p.889, Abstract 3852 (poster session)

PERSONNEL

Full-time personnel who received pay from this research effort include:

Church, Dawn R.
Jarrard, David F.
Thompson, Todd A.
Wilding, George
Zhang, Ying
Zhong, Weixiong

Appendices

Appendix 1

ACRONYMS AND ABBREVIATIONS

4HNE	4-hydroxy-2-nonenal
AP-1	activator protein 1
AR	androgen receptor
ATCC	American Type Culture Collection
ATS	alpha-tocopheryl succinate
BHA	butylated hydroxyanisole
CAT	catalase
cDNA	complimentary deoxyribonucleic acid
CMV	cytomegalovirus
Cu/ZnSOD	copper/zinc superoxide dismutase
DCF	2',7'-dichlorodihydrofluorescein diacetate
DHT	5 α-dihydrotestosterone
DNA	deoxyribonucleic acid
DU145	Duke University 145; an established prostate cancer cell line
ECL	Enhanced Chemiluminescence
EGFP	enhanced green fluorescence protein
EMSA	electrophoretic mobility shift assay
ETS	electron transport system
G418	geneticin
GFP	green fluorescence protein
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HPECs	human prostate epithelial cells
HPLC	high performance liquid chromatography
IκB	inhibitor factor kappa-B
IRES	internal ribosomal entry site
LAPC4	an established prostate cancer cell line
LNCaP	lymph node cancer prostate; an established prostate cancer cell line
MDA	malondialdehyde
μM	micromolar
mM	millimolar
MMTV	mouse mammary tumor virus
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	nuclear factor kappaB
nM	nanomolar

OH	hydroxy
PC3	prostate cancer 3; an established prostate cancer cell line
PSA	prostate specific antigen
pSVneo	plasmid SV40 promoter, neomycin resistance expression vector
qPCR	quantitative real time polymerase chain reaction
R1881	methyltrienolone
RLU	relative luciferase units
RNA	ribonucleic acid
ROS	reactive oxygen species
TK	thymidine kinase
TNF-α	tumor necrosis factor alpha
U.S.	United States
UW	University of Wisconsin - Madison
UWCCC	University of Wisconsin Comprehensive Cancer Center

Appendix 2, Figure 1

a.

$[R1881]$ (nM)

| 0 | 1.0 | 0.1 |

IκBα

b.

Relative Intensity (x10^6)

$[R1881]$ (nM)

c.

$[R1881]$ (nM)

| 0 | 1.0 | 0.1 |

IκBβ
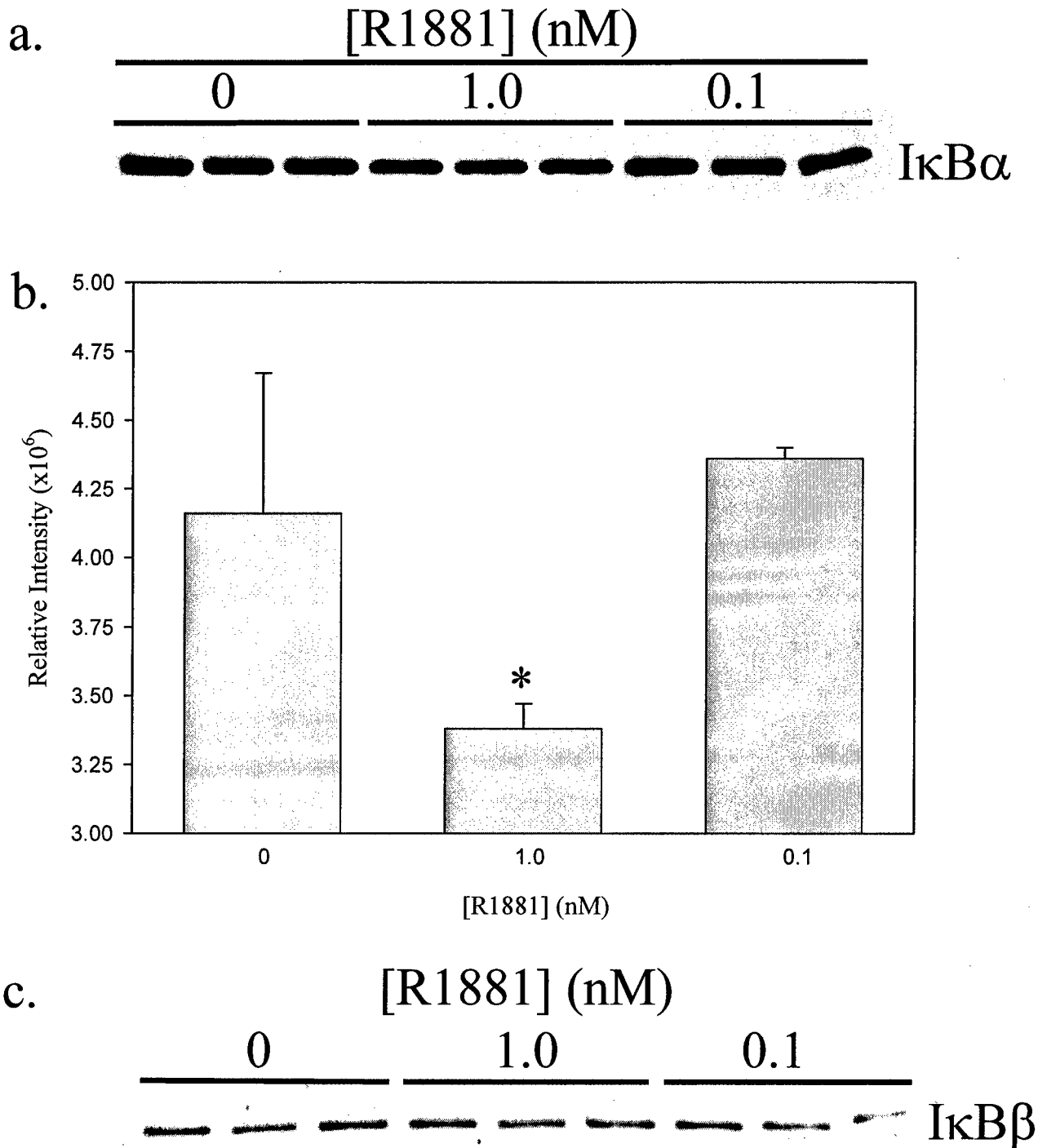
Figure 1. Analysis of IκBα and IκBβ protein expression in LNCaP cells exposed to 0, 1.0, or 0.1 nM R1881 for 4 days. (a.) IκBα protein levels were measured by Western analysis. Three samples each from LNCaP cells exposed to 0, 1.0, or 0.1 nM R1881 were analyzed. (b.) Band intensities were determined using ImageQuant software from ECL Plus developed blots. Cells exposed to 1.0 nM R1881 were found to have a significant reduction in IκBα protein levels (*$P<0.05$). (c) IκBβ protein levels were measured by Western analysis. No significant difference in protein levels were found for cells exposed to 0, 1.0, or 0.1 nM R1881 for 4 days.

Appendix 2, Figure 2
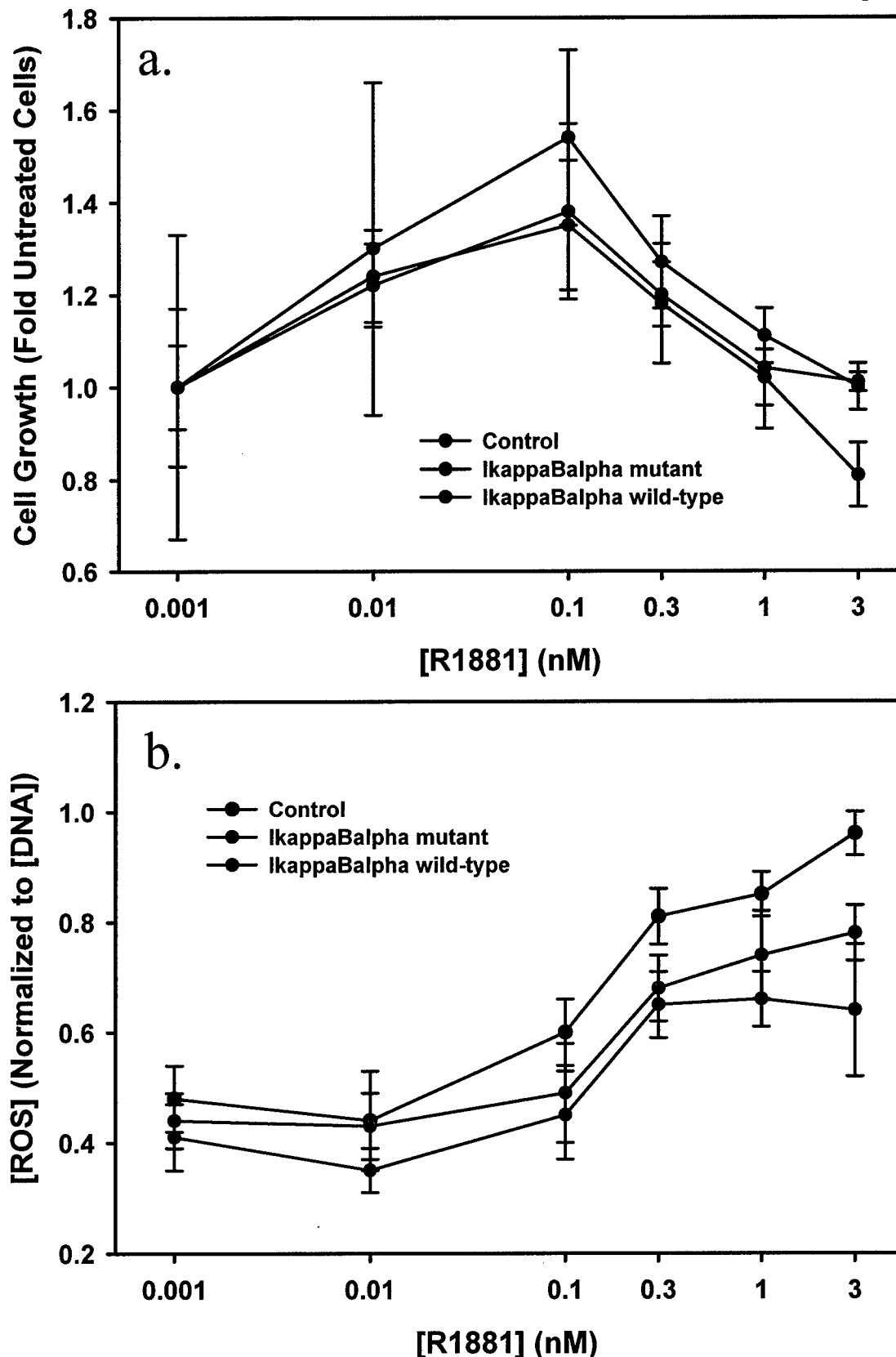
Figure 2. Determination of androgen-induced growth modulation (a.) and ROS production (b.) in LNCaP cells stably expressing a mutant form of IkappaBalpha that inhibits NFκB translocation. Cells stably expressing a wild-type IkappaBalpha were used as controls. Inhibition of NFκB activity (i.e., cells expressing the IkappaBalpha mutant) did not affect either androgen-induced growth arrest or ROS production compared to control cells.

Appendix 2, Figure 3

pIRES2-EGFP Vector
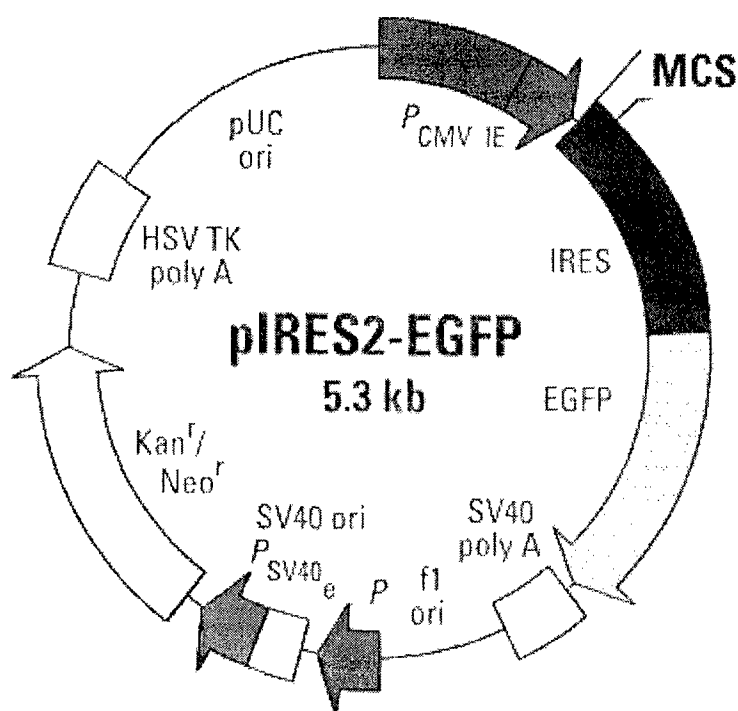
Figure 3. Schematic of the pIRES2-EFGP expression vector (Clontech/BD Biosciences) used in many of the studies performed in this project. Genes of interest (e.g., *jun*D, *jun*DdeltaTA, and the androgen receptor) were subcloned in to the multiple cloning site (MCS) of pIRES2-EFGP for use in these studies. All plasmids used in these studies were purified using the Plasmid Midi Kit (QIAGEN).

Cell DNA Content

Reactive Oxygen Species Production
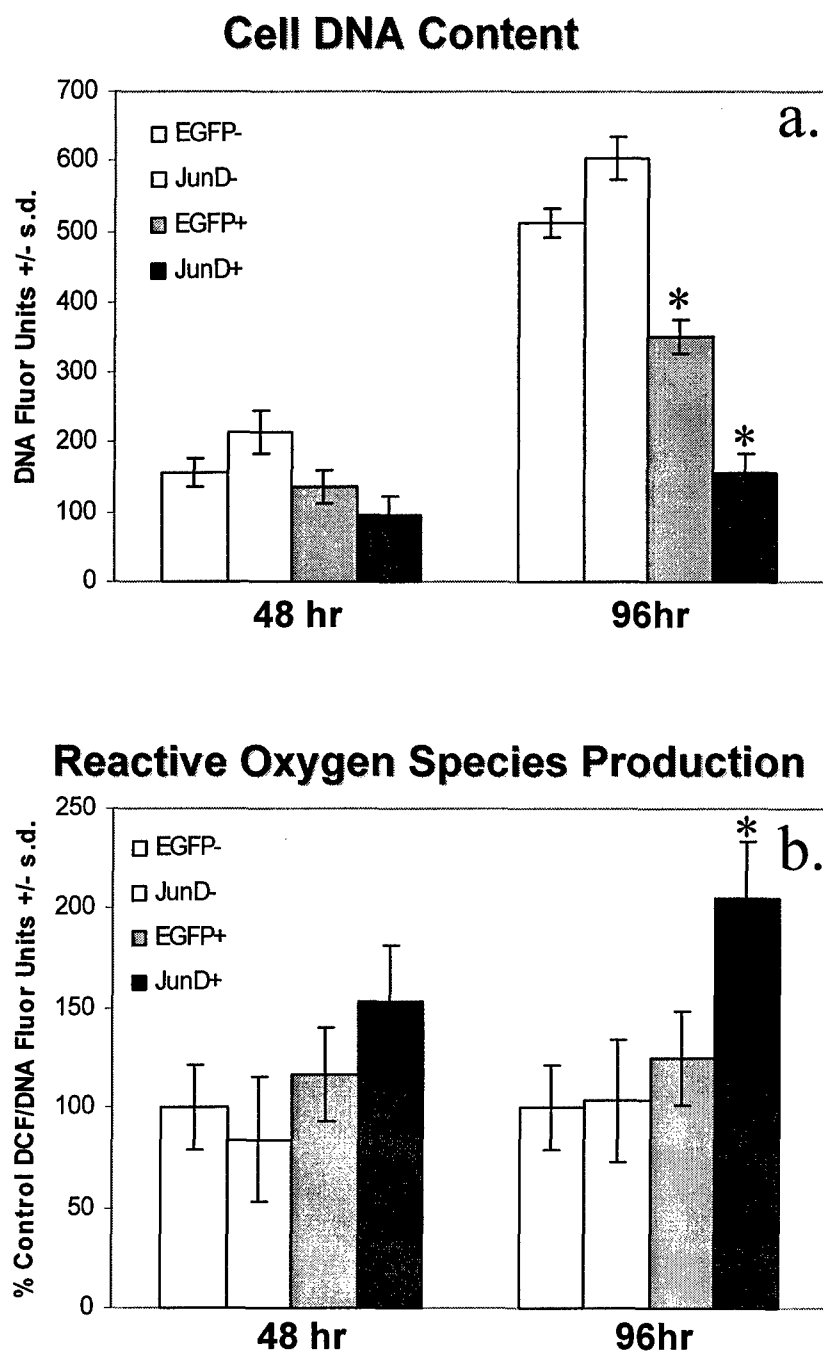
Figure 4. Cell growth inhibition (a.) and increased ROS production (b.) in LNCaP cells expressing JunD. LNCaP cells were transiently transfected using Effectene (Qiagen) with a vector co-expressing EGFP and JunD or a vector expressing only EGFP. Cells were sorted into 96-well plates using a fluorescence activated cell sorter (Becton-Dickinson). At 48 and 96 hours after sorting of negative populations (i.e., EFGP- and JunD-) and positive populations (i.e., EGFP+ and JunD+) the cell number was determined using a Hoechst dye based assay (a.). ROS production was determined using a DCF assay and normalized for cell number (b.). At 96 hours, JunD expression was found to significantly inhibit cell growth and increase ROS production. * $P<0.05$ compared to EGFP- and JunD- cells.

Appendix 2, Figure 5

a. **pIRES2.EGFP (Control expression vector)**

CMV	MCS	IRES	EGFP	SV40 polyA

pJunDΔTA (JunD dominant negative expression vector)

CMV	JunDΔTA	IRES	EGFP	SV40 polyA

b.

~42kD
~30kD

JunDΔTA
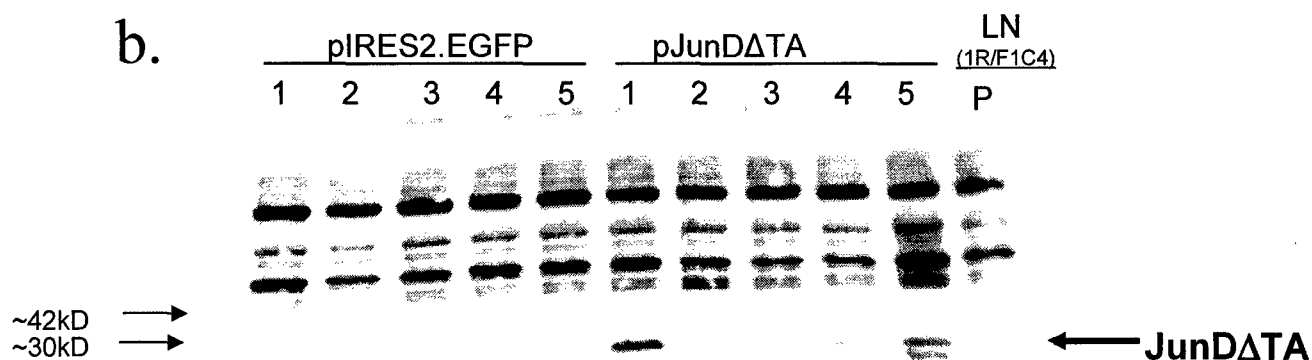
Figure 5. Schematic of JunDΔTA construct and expression in LNCaP cells. (a) To construct pJunDΔTA, dominant-negative *jun*D was placed in the multiple cloning site of pIRES2.EGFP (diagram not to scale - see Figure 3). (b) Five LNCaP clones were stably transfected with the backbone vector pIRES2.EGFP (1-5) and 5 clones stably transfected with pJunDΔTA (1-5) after selection with G418. Nuclear protein extracts were screened for JunD protein expression by immunoblotting and compared to nuclear protein extracted from untransfected LNCaP cells (LN). JunD protein expression from pIRES2.EGFP nuclear extracts were equivalent to untransfected LNCaP cells. pJunDΔTA clones 1, 3, 4, and 5 showed variable levels of JunDΔTA protein expression (JunDΔTA); pJunDΔTA clone 2 did not show demonstrable JunDΔTA protein expression.
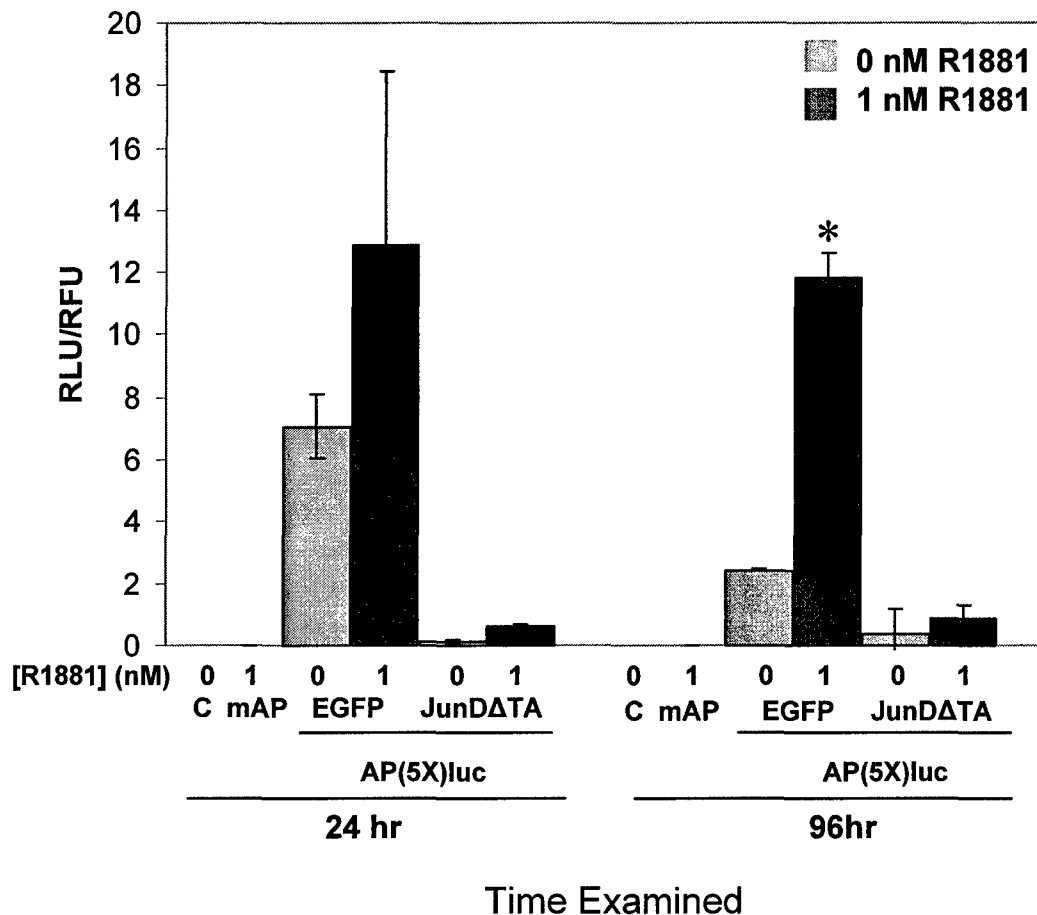
Figure 6. Inhibition of AP-1 induction by androgen in LNCaP cells expressing dominant-negative JunD (i.e., JunDΔTA). Untreated controls (C) and cells transfected with an expression vector containing a mutant AP-1 site (mAP), control cells expressing EGFP (transfected with pIRES2.EGFP), and cells transfected with a vector expressing *jun*DΔTA were examined for luciferase expression driven by an AP-1 consensus sequence (AP(5X)luc) at 24 and 96 hr after 1 nM R1881 exposure. JunDΔTA expression significantly inhibited AP-1 activation by androgen. Luciferase expression levels are expressed as relative luciferase units (RFU), which were normalized to the expression levels of a TKβgal vector (RFU). AP-1 expression was stimulated by 1 nM R1881 exposure in cells expressing the control vector, whereas cells expressing JunDΔTA did not show stimulation by exposure to 1 nM R1881. * *P*<0.05 – 0 compared to 1 nM R1881 exposure.

Appendix 2, Figure 7

a.
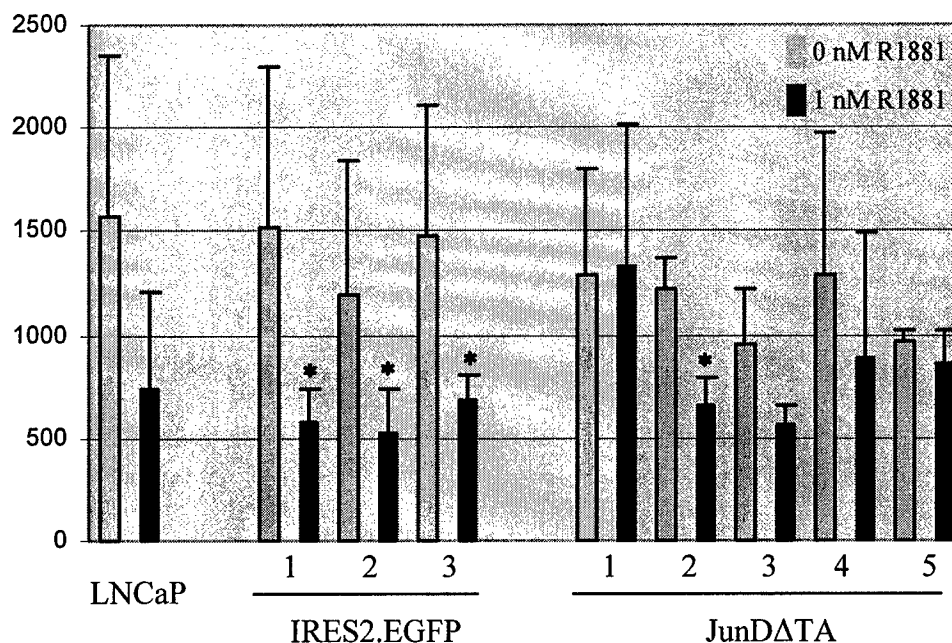
b.
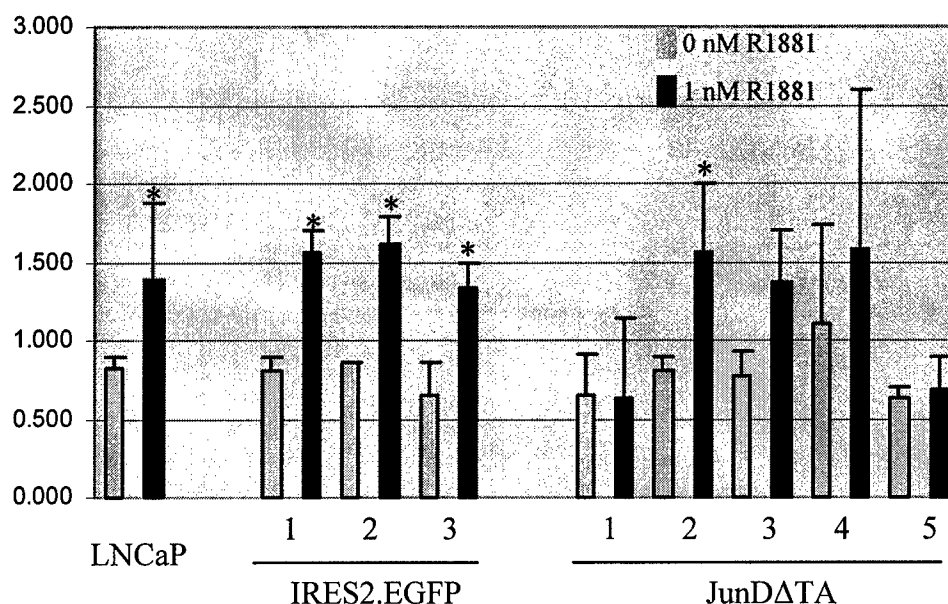
Figure 7. Growth inhibitory response and ROS production in LNCaP clones stably transfected with pIRES2.EGFP or pJunDΔTA, with and without 1 nM R1881 exposure. (a) Exposure to 1 nM R1881 significantly inhibited the growth of control IRES2.EGFP clones 1, 2, and 3 as well as JunDΔTA clone 2. (b) One nM R1881 exposure resulted in signifcant ROS production (measured using the DCF assay) in all IRES2.EGFP clones and in JunDΔTA clone 2 (JunDΔTA clone 2 did not show demonstrable JunDΔTA protein expression – see Figure 5). * $P<0.05$ compared to 0 nM R1881 exposure.

Appendix 2, Table 1

Table 1. Microarray analysis of mRNA isolated from control and 1 nM R1881 exposed LNCAP cells.

Name	Probe #	Identifier	Control	1nM R1881	Fold Change*
Catalase	201432_at	NM_001752	3275	1876	-1.8
Cu/ZnSOD	200642 _at	NM_00045	5590	5866	NC (1.1)
MnSOD	216841 _at	X15132	205	163	NC (-1.3)
Heme Oxygenase 1	203665 _at	NM_002133	171	5521	32.3
Glutathione Peroxidase 1	200736_s_	NM_00058	4121	4581	NC (1.1)
Glut. Per. 2	202831_at	NM_00208.1	379	436	NC (1.2)
Glut. Per. 3	201348_at	NM_00208.2	80	59	NC (-1.4)
Glut. Per. 4	201106_at	NM_00208.3	4911	5984	NC (1.2)
Glut. Per. 5	208028_s_	NM_00399	408	380	NC (-1.1)
Peroxi-redoxin 1	208680_at	L19184.1	7939	5469	-1.5
Peroxired. 2	201006_at	NM_00580	201	138	-1.5
Peroxired. 3	209766_at	AF118073	139	166	NC (1.2)
Peroxired. 4	201923_at	NM_00640	3376	4982	1.5
Thioredoxin	208864_s_	AF313911	3603	2923	NC (-1.2)
Thioredoxin Reductase 1	201266_at	NM_00333	1954	5540	2.8
Thioredoxin Reductase 2	221906_at	BF513089	328	228	NC (-1.4)

The microarray analysis was performed using the Affymetrix U133A chip. * NC – No change – a fold change in mRNA between -1.5 and 1.5 was considered questionable. Notably, the oxidative stress responsive heme oxygenase 1 transcript was increased 32.3-fold by 1 nM R1881 exposure.

Appendix 2, Figure 8

1 2 3 4 5 6 7 8 9 10 11 12

MnSOD

CuZnSOD

Catalase

β - Actin

1 nM R1881   - + - +   - + - +   - + - +
Time (hr)   24   96   24   96   24   96
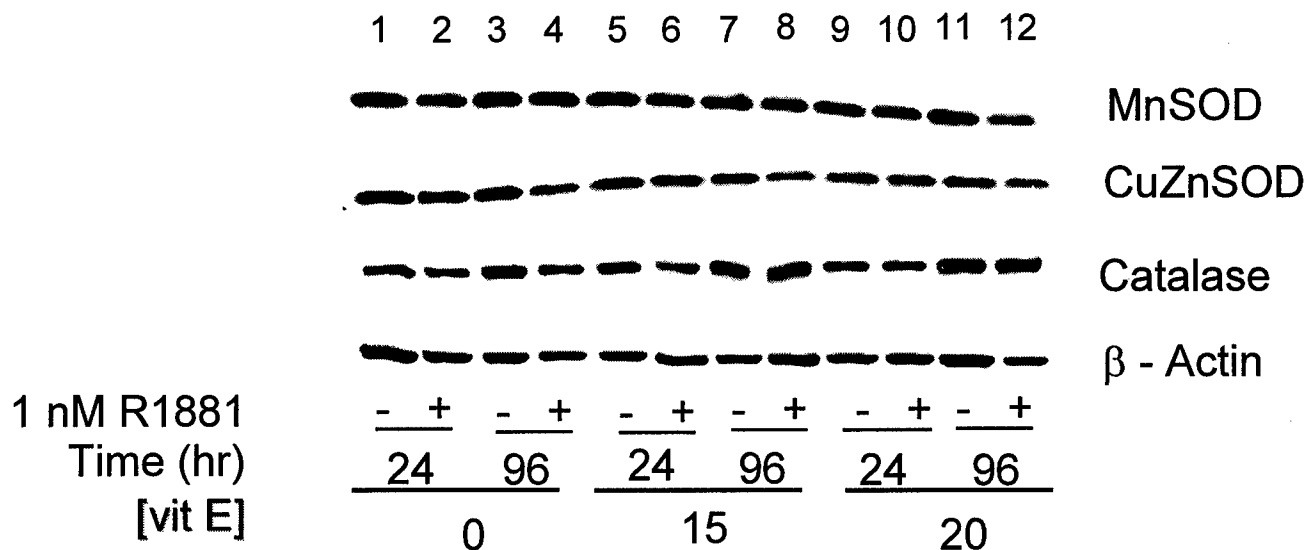[vit E]   0   15   20

Figure 8. Effect of vitamin E succinate on antioxidant enzyme immunoreactive protein levels. LNCaP cells were cultured in 4 % CSS and treated with vitamin E succinate (15 or 20 μM) in vehicle control medium (containing 0.01% ethanol) or 1 nM R1881 for 24 or 96 hours. The protein level of MnSOD, Cu/ZnSOD, and catalase was analyzed by immunoblotting. Total proteins (10 μg for MnSOD and actin, 20 μg for Cu/ZnSOD and catalase) from each treatment were separated on a 12.5% SDS-PAG and electrotransfered onto a nitorcellulose membrane. The blots were probed with primary MnSOD, CuZnSOD, catalase and β-actin antibody, respectively, followed by a horseradish peroxidase-linked secondary antibody. The bands corresponding to MnSOD, Cu/ZnSOD, and catalase were detected using ECL plus. Actin was used for normalizing loading levels. No changes in immunoreactive protein were detected at the time points or treatments for any of the immunoreactive proteins.

Appendix 2, Tables 2 and 3

Table 2. Antioxidant enzyme activities at 24 hours in EtOH control, R1881, and vitamin E succinate treated cells.

Cells	CuZnSOD[a] (units/mg protein)	MnSOD[a] (units/mg protein)	CAT[b] (k/g protein)	GPx[a] (units/mg protein)
EtOH	143 ± 4	85 ± 4	5246 ± 55	41 ± 1
0.05R	158 ± 8	83 ± 5	4977 ± 36	42 ± 3
1R	148 ± 5	83 ± 3	5542 ± 160	36 ± 3
20E	134 ± 2	97 ± 4	7480 ± 219 [c]	37 ± 1
0.05R20E	160 ± 3	91 ± 4	5800 ± 186	40 ± 1
1R20E	128 ± 7	87 ± 4	2915 ± 44 [c]	38 ± 2

[a] Results are expressed as mean $\pm$ SE from five independent samples.
[b] Results are expressed as mean $\pm$ SE from three independent samples.
[c] $p < 0.05$ compared to EtOH control cells.

Table 3. Antioxidant enzyme activities at 96 hours in EtOH control, R1881, and vitamin E succinate treated cells.

Cells	CuZnSOD[a] (units/mg protein)	MnSOD[a] (units/mg protein)	CAT[b] (k/g protein)	GPx[a] (units/mg protein)
EtOH	147 ± 2	124 ± 6	3962 ± 73	41 ± 1
0.05R	128 ± 6	137 ± 6	4655 ± 187[c]	9 ± 1 [c]
1R	146 ± 2	195 ± 9 [c]	4211 ± 103	29 ± 1 [c]
20E	158 ± 10	51 ± 4 [c]	298 ± 36 [c]	15 ± 1 [c]
0.05R20E	127 ± 6	121 ± 4	1497 ± 58 [c]	26 ± 2 [c]
1R20E	116 ± 2	230 ± 12 [c]	348 ± 25 [c]	7 ± 1 [c]

[a] Results are expressed as mean $\pm$ SE from five independent samples.
[b] Results are expressed as mean $\pm$ SE from three independent samples.
[c] $p < 0.05$ compared to EtOH control cells.
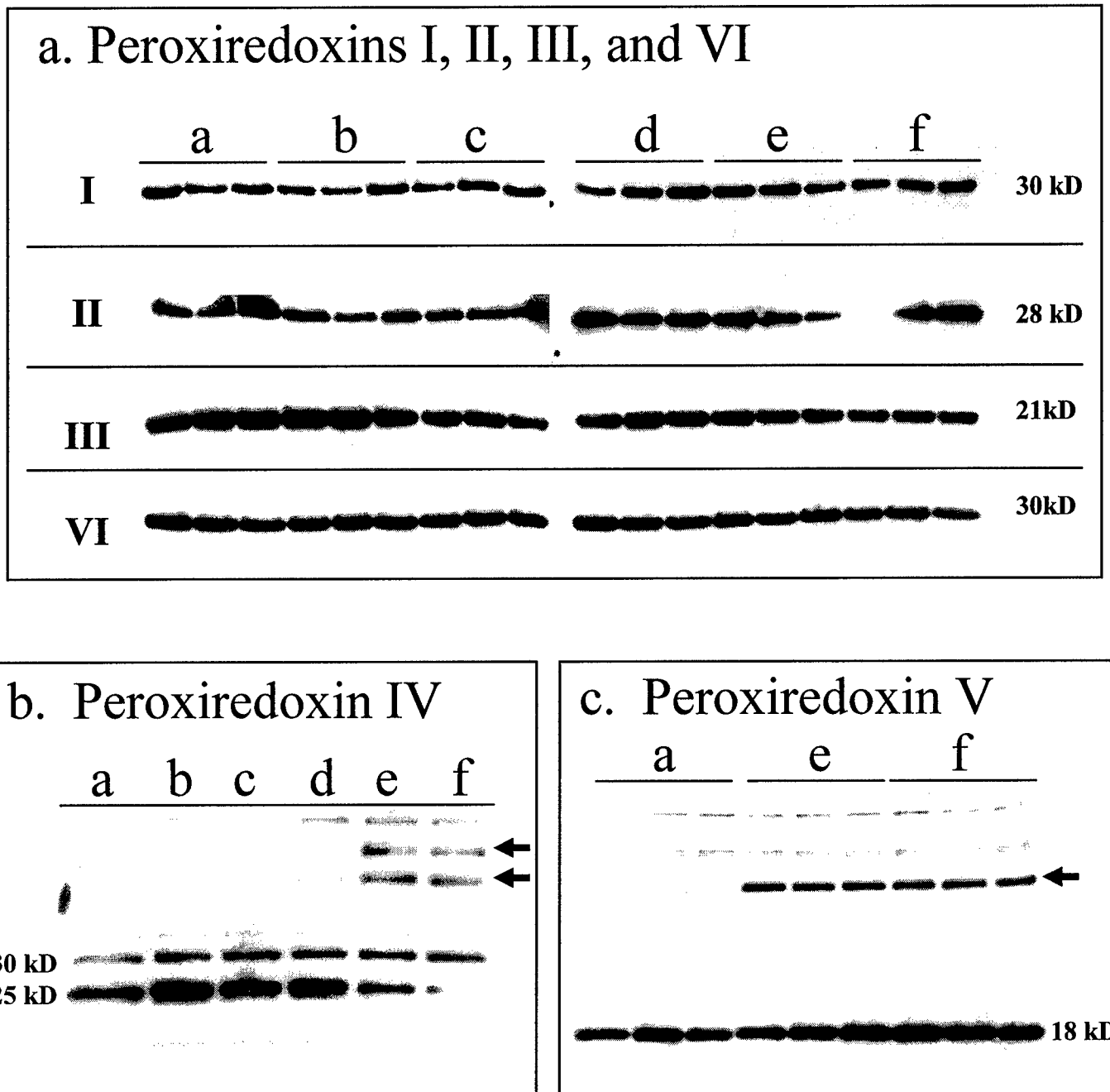
Figure 9. Immunoblot analysis of peroxiredoxin I, II, III, IV, V, and VI protein levels after exposure to androgen and vitamin E. Immunoblot analysis were performed as described in Figure 8 using primary antibodies for peroxiredoxin I, II, III, IV, V, and VI proteins. (a) No changes in the levels of peroxiredoxin I, II, III and VI proteins after exposure to 0.05 or 1.0 nM R1881 or 20 μM vitamin E succinate for 4 days. (b) High MW peroxiredoxin IV protein bands were increased in LNCaP cells exposed to 1 nM R1881 with or without 20 μM vitamin E succinate for 4 days. (c) Although the prominent 18 kD peroxiredoxin V protein band was not affected by androgen or vitamin E treated a high MW band was found to be more prominent in LNCaP cells exposed to 1 nM R1881 with or without vitamin E succinate treatment. *a*, control vehicle-treated cells; *b*, 20 μM vitamin E succinate; *c*, 0.05 nM R1881; *d*, 0.05 nM R1881 + 20 μM vitamin E succinate; *e*, 1.0 nM R1881; *f*, 1.0 nM R1881 + 20 μM vitamin E succinate.

Appendix 2, Figure 10
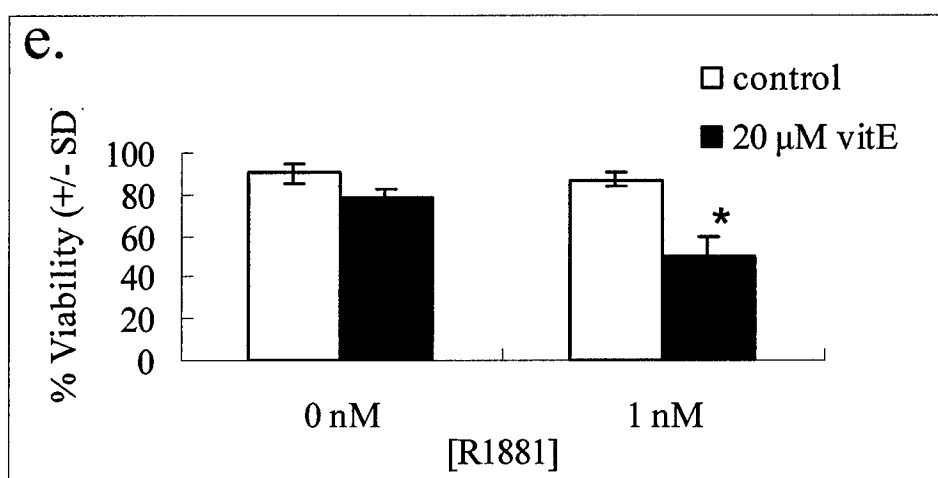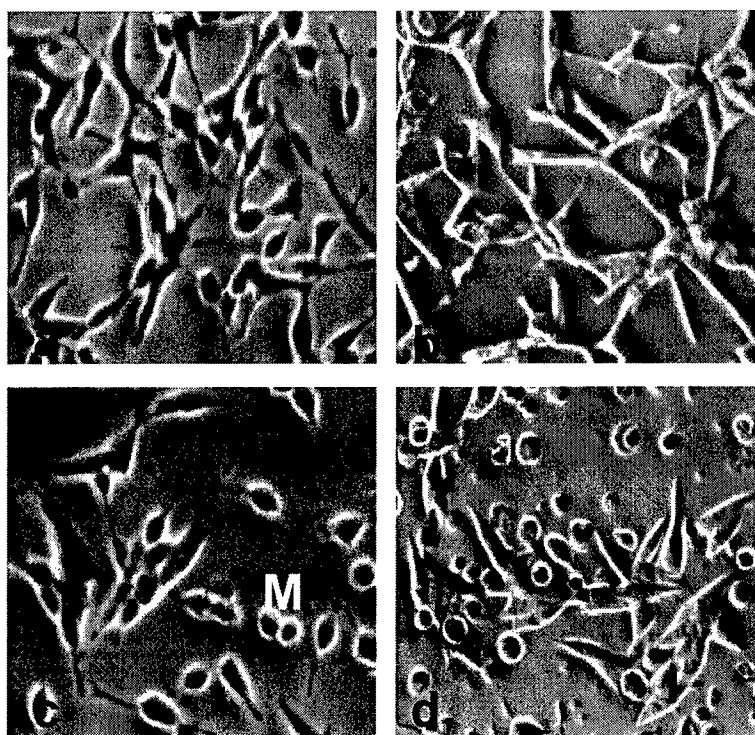
Figure 10. Phase contrast microscopy and viability analysis of LNCaP cells treated androgen and vitamin E succinate. Phase contrast morphological analysis of LNCaP cells treated for 4 days with: (a) EtOH vehicle; (b) 1 nM R1881; (c) 20 μM vitamin E succinate; (d) 1 nM R1881 + 20 μM vitamin E succinate (× 200 magnification). Mitosis (*M*) observed in (c) can be distinguished from cell death in (d). Mitotic cells were larger and adjacent to each other, while apoptotic cells were smaller and were single cells. (e) LNCaP cells treated with androgen or vitamin E succinate only did not show an effect on viability as measured by trypan blue exclusion. The co-administration of 1 nM R1881 + 20 μM vitamin E succinate produced a significant decrease in cell viability (mean ± SE; n = 3; *P<0.05).

Cell Count
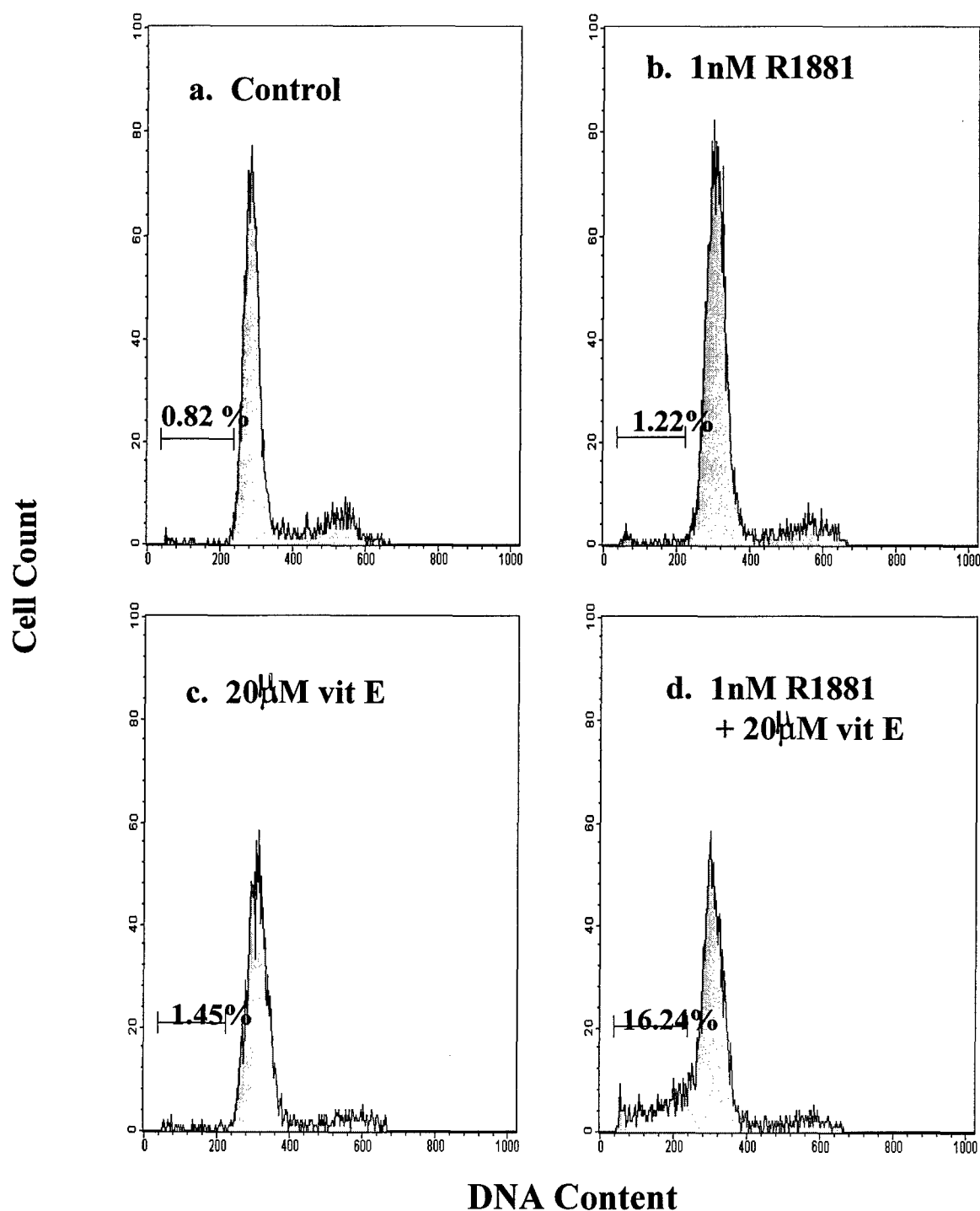
DNA Content

Figure 11. Hypodiploid analysis of LNCaP cells after esxposure to androgen and vitamin E for 96 hours. Cells were treated with: (a) EtOH; (b) 1 nM R1881; (c) 20 μM vitamin E succinate; (d) 1 nM R1881 + 20 μM vitamin E succinate. DNA content was determined by propidium iodide staining and analyzed by flow cytometry. The percentage of apoptotic cells is indicated by the hypodiploid (sub-G1) peak. Only cells co-treated with 1 nM R1881 and 20 μM vitamin E succinate showed a significant hypodiploid fraction.

Appendix 2, Figure 12
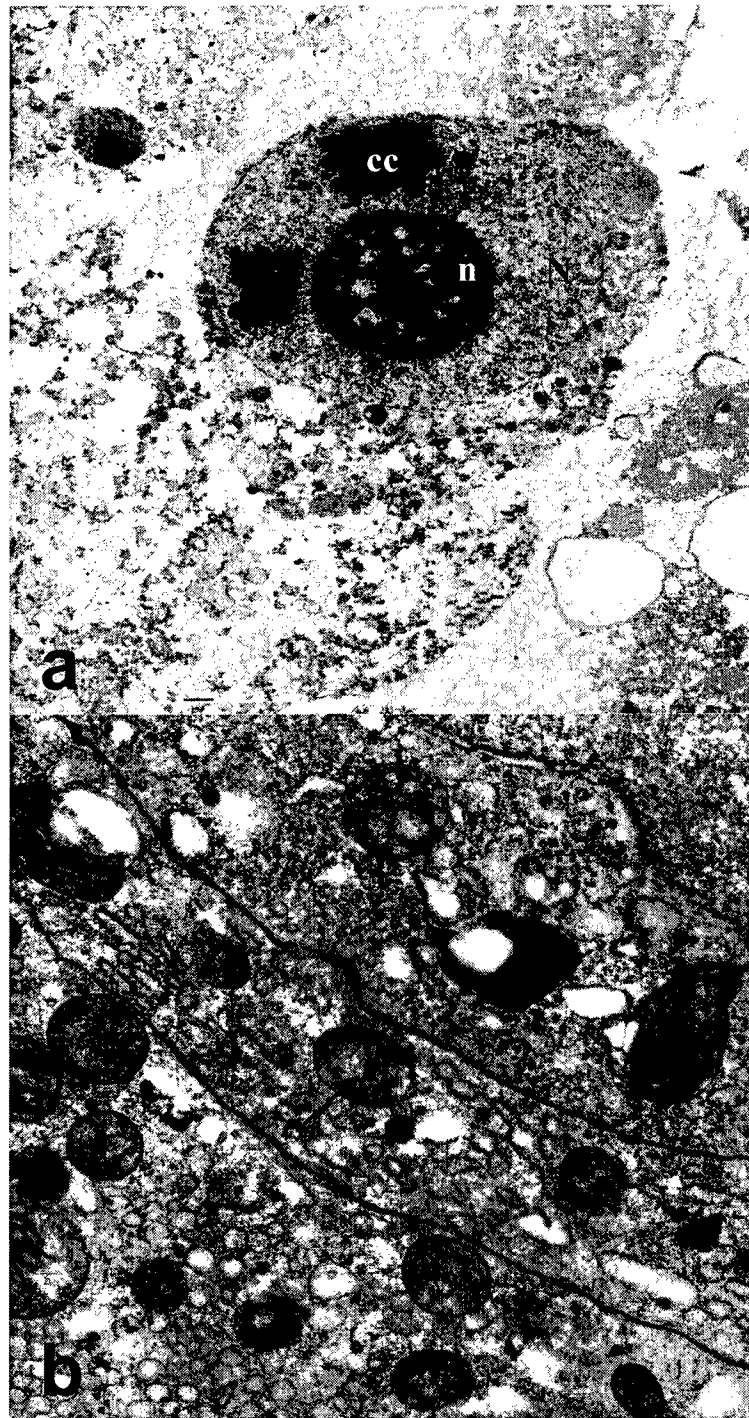
Figure 12. Electron microscopy showing effects of androgen and vitamin E on cell morphology. (a) Floating cells treated with the combination of 1 nM R1881 and 20 μM vitamin E succinate (× 5,200); *cc*, chromatin condensation; *n*, nucleolus; *N*, nucleus. (b) Cells on the culture substrate treated with the combination of 1 nM R1881 and 20 μM vitamin E succinate (× 15,100). Floating cells showed nuclear fragments with prominent chromatin condensation while attached cells showed mild mitochondria injury with focal loss of cristae (arrow).

a.

* $p < 0.05$ compared to EtOH control cells.

b.
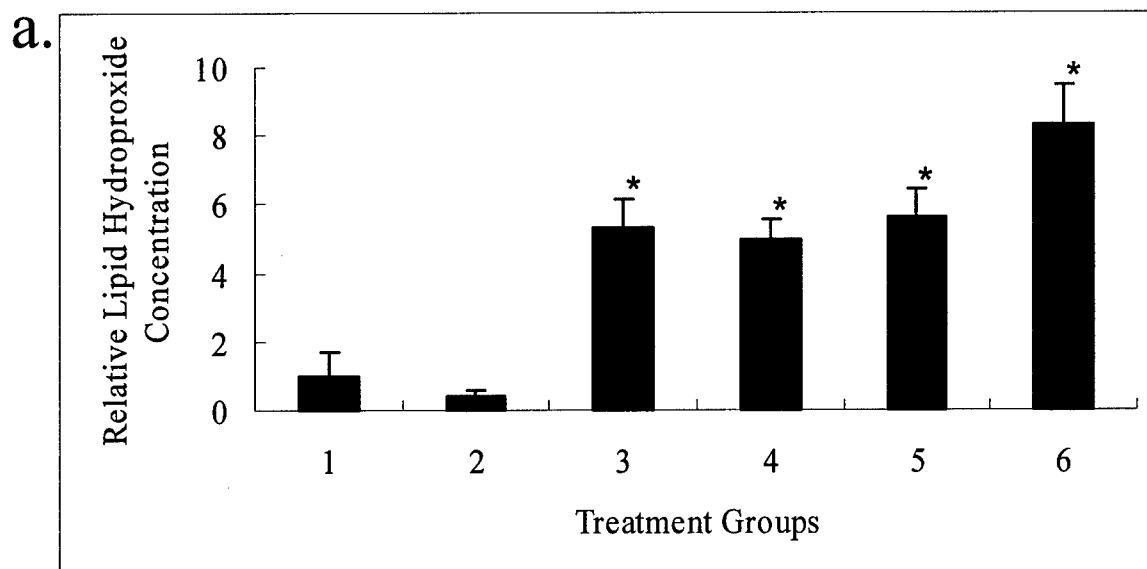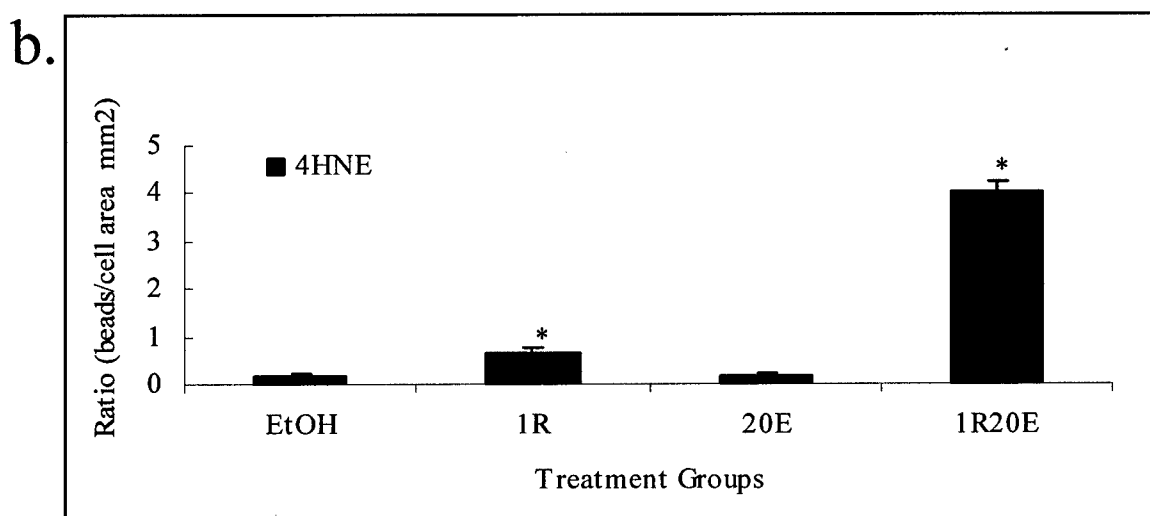
* $p < 0.05$ compared to EtOH control cells.

Figure 13. Effect of androgen and vitamin E succinate on lipid peroxidation and 4-hydroxy-2-nonenal protein adducts. (a) The concentration of lipid hydroperoxides were measured in LNCaP cells treated with androgen and vitamin E succinate for 96 hours. 1, EtOH; 2, 0.05 nM R1881; 1.0 nM R1881; 4, 20 μM vitamin E succinate; 5, 0.05 nM R1881 + 20 μM vitamin E succinate; 6, 1.0 nM R1881 + 20 μM vitamin E succinate. Mean ± SE; n = 3; *$P<0.05$ compared to controls at 96 hours. (b) Ratio of 4HNE-protein/cell area (mm^2) in cells treated with control, 1 nM R1881, 20 μM vitamin E succinate, and the combination of 1 nM R1881 + 20 μM vitamin E succinate. LNCaP cells treated with either 1 nM R1881 or the combination of 1 nM R1881 + 20 μM vitamin E succinate had significantly higher levels of 4HNE protein adducts. *$P<0.05$.
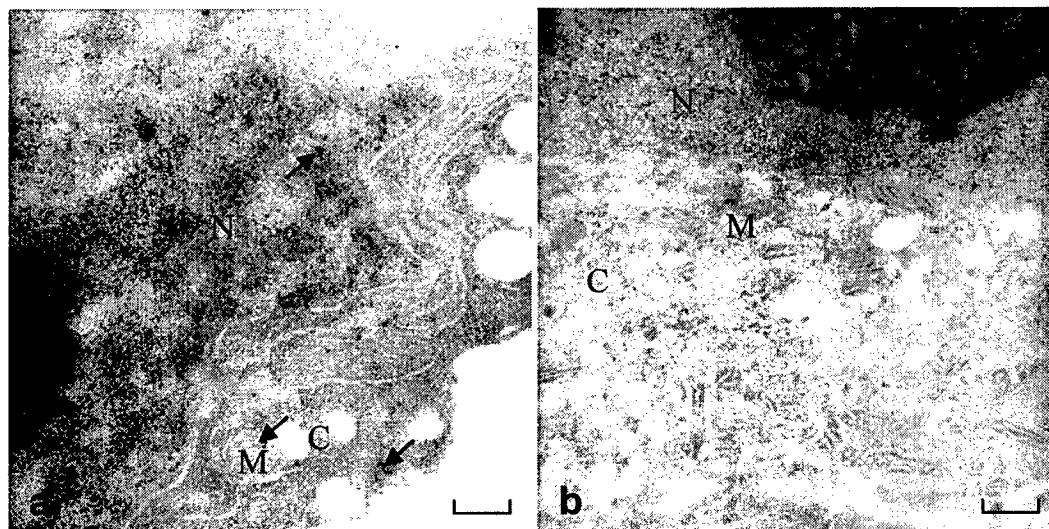
Figure 14. Immunogold microscopy analysis of oxidative damage product 4-HNE-protein adducts in androgen and vitamin E succinate treated LNCaP cells at 96 hours. (a) Immunogold electron microscopy of 4HNE protein adducts in cells treated with 1 nM R1881 and 20 μM vitamin E succinate. M, mitochondrion; N, nucleus; C, cytoplasm. (b) Immunogold electron microscopy of 4HNE protein adducts in control cells. N, nucleus; n, nucleolus; C, cytoplasm. In cells treated with 1 nM R1881 and 20 μM vitamin E succinate, high levels of 4HNE protein adducts are identified in nuclei, cytoplasm, and mitochondria. Untreated control cells had only rare gold beads.

Table 4. Summary of statistical analyses of lipid hydroperoxide and 4-hydroxy-2-nonenal results for LNCaP cells after androgen and vitamin E succinate treatment.

	EtOH	0.05R	1R	20E	0.05R20E	1R20E
EtOH	—	N	a	a	a	a, b
0.05R	N	—	a	a	a	a
1R	a, b	a	—	b	N	a, b
20E	a	a	b	—	N	a, b
0.05R20E	a	a	N	N	—	a
1R20E	a, b	a	a, b	a, b	a	—

[a] $p < 0.05$ compared with control at 96 hours for LPO;
[b] $p < 0.05$ compared with control at 96 hours for 4HNE;
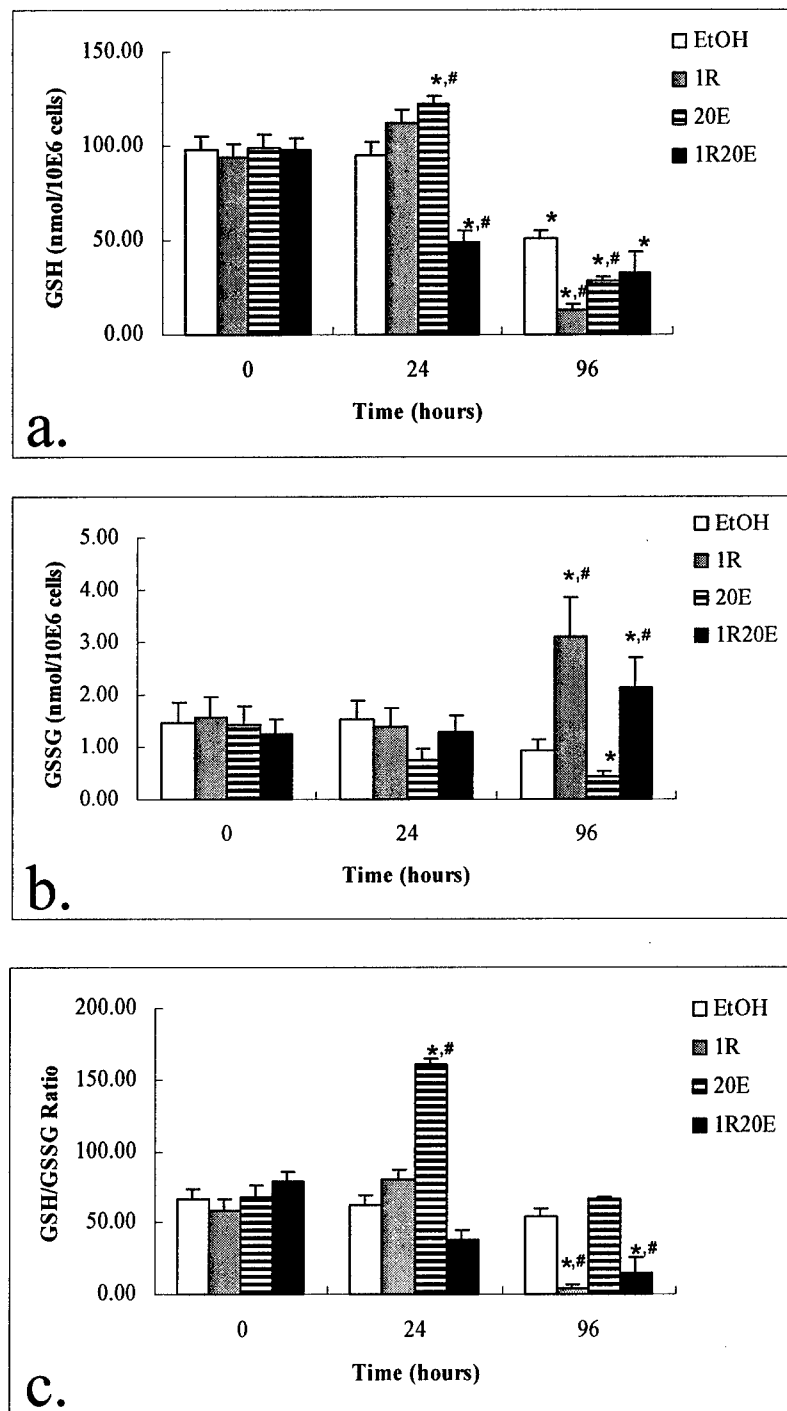N, not statistically significant.

Figure 15. Effect of androgens and vitamin E succinate on intracellular GSH, GSSG, GSH/GSSG ratios at 24 and 96 hours in LNCaP cells measured using the Tietze assay. (a) Reduced glutathione (GSH) levels were decreased in all cells at 96 hours, particularly in cells exposed to 1 nM R1881. (b) Oxidized glutathione (GSSG) levels were increased in cells exposed to 1 nM R1881 for 96 hours, with or without vitamin E succinate treatment. (c) The ratio of GSH/GSSG was significantly decreased in cells exposed to 1 nM R1881, with or without vitamin E succinate treatment. *$P< 0.05$ compared to EtOH control cells at 0 hour; # $P < 0.05$ compared to EtOH cells at the same time points
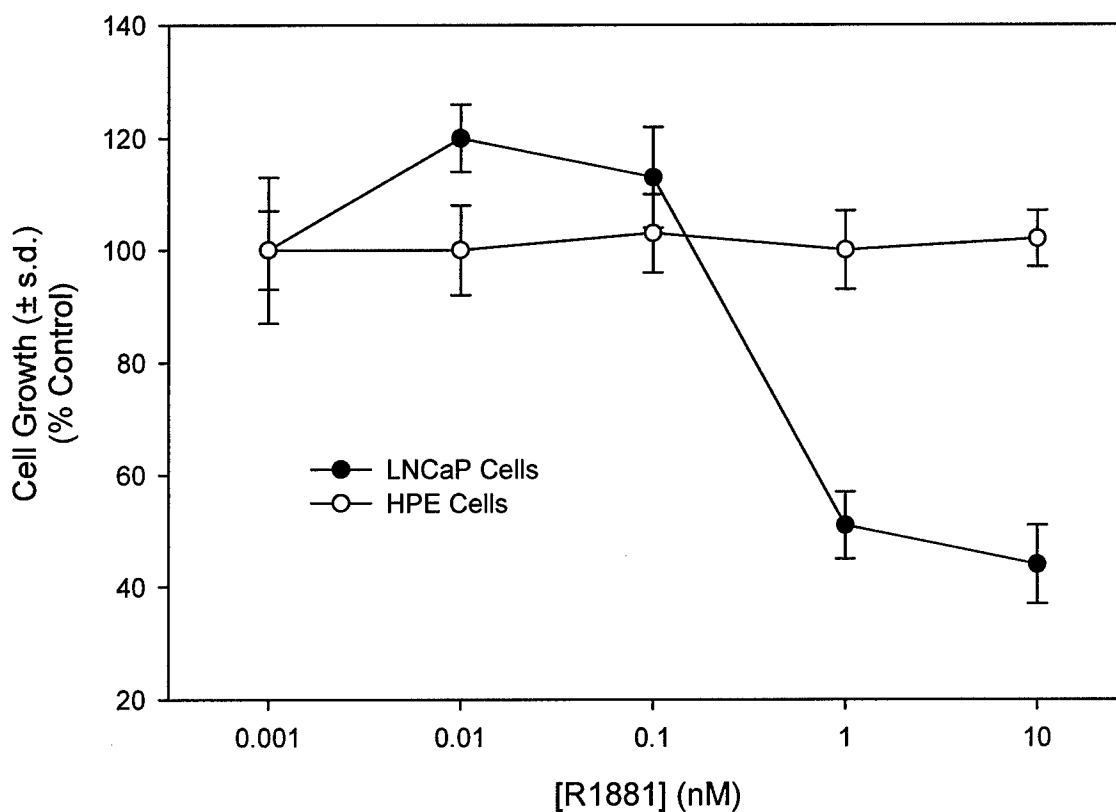
Figure 16. R1881 dose-response of 15E6 human prostate epithelial cell (HPEC) growth compared to LNCaP cell growth. Immortalized HPECs were acquired from Dr. Jarrard's laboratory and grown as described (Jarrard et al., 1999). Similary, 15E7 HPECs acquired from Dr. Jarrard's laboratory were not responsive to androgen exposure (data not shown). Cell growth was determined as previously described (Ripple et al., 1997).
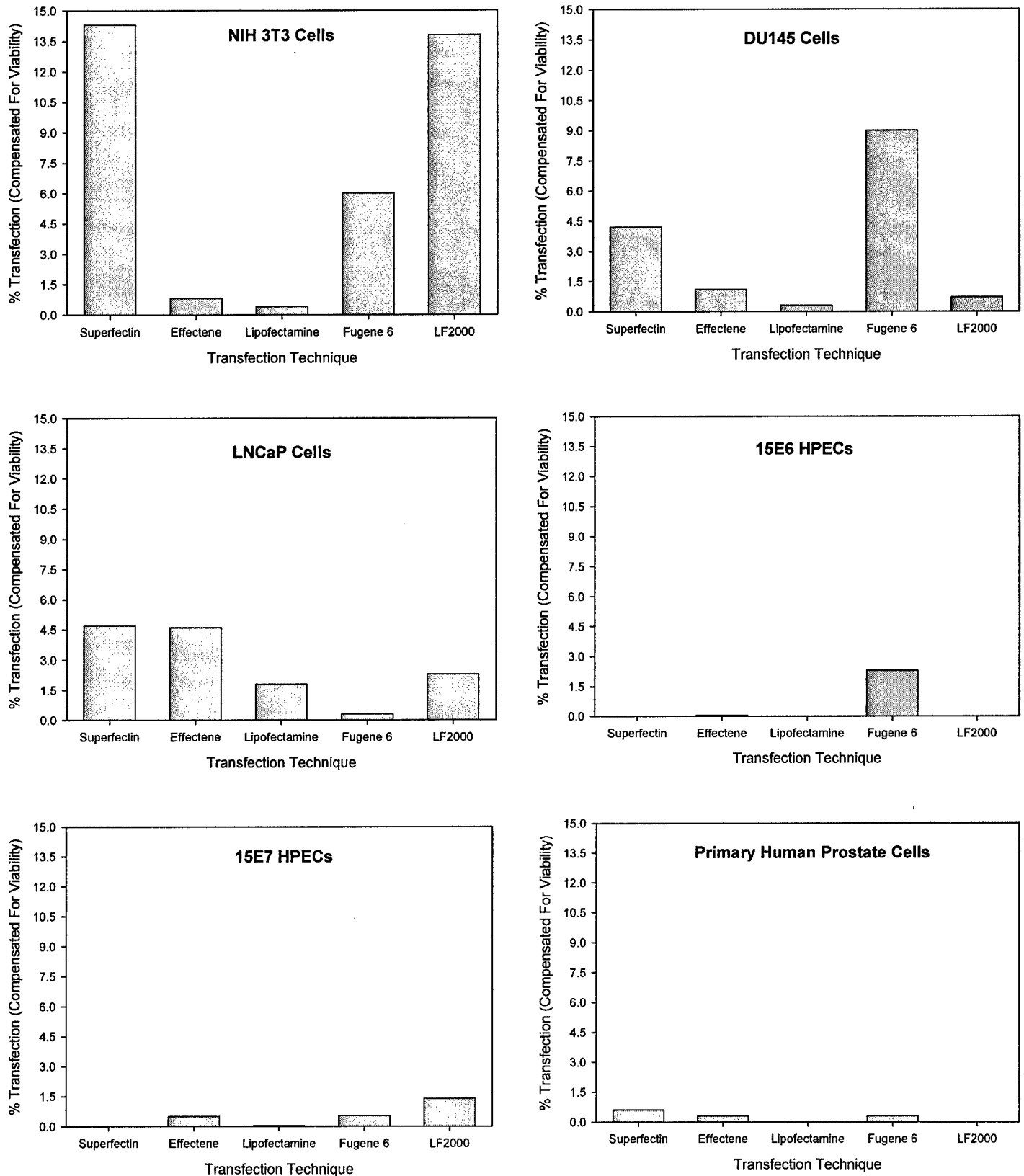
Figure 17. Efficiency of CMVβgal plasmid transduction using various chemically mediated transfection methods. Although some of the methods used effectively transduced NIH3T3 fibroblast cells, DU145, and LNCaP human prostate carcinoma cells, they worked poorly to transduce 15E6, 15E7, and primary human prostate cells. (LF2000 = Lipofectamine 2000.)
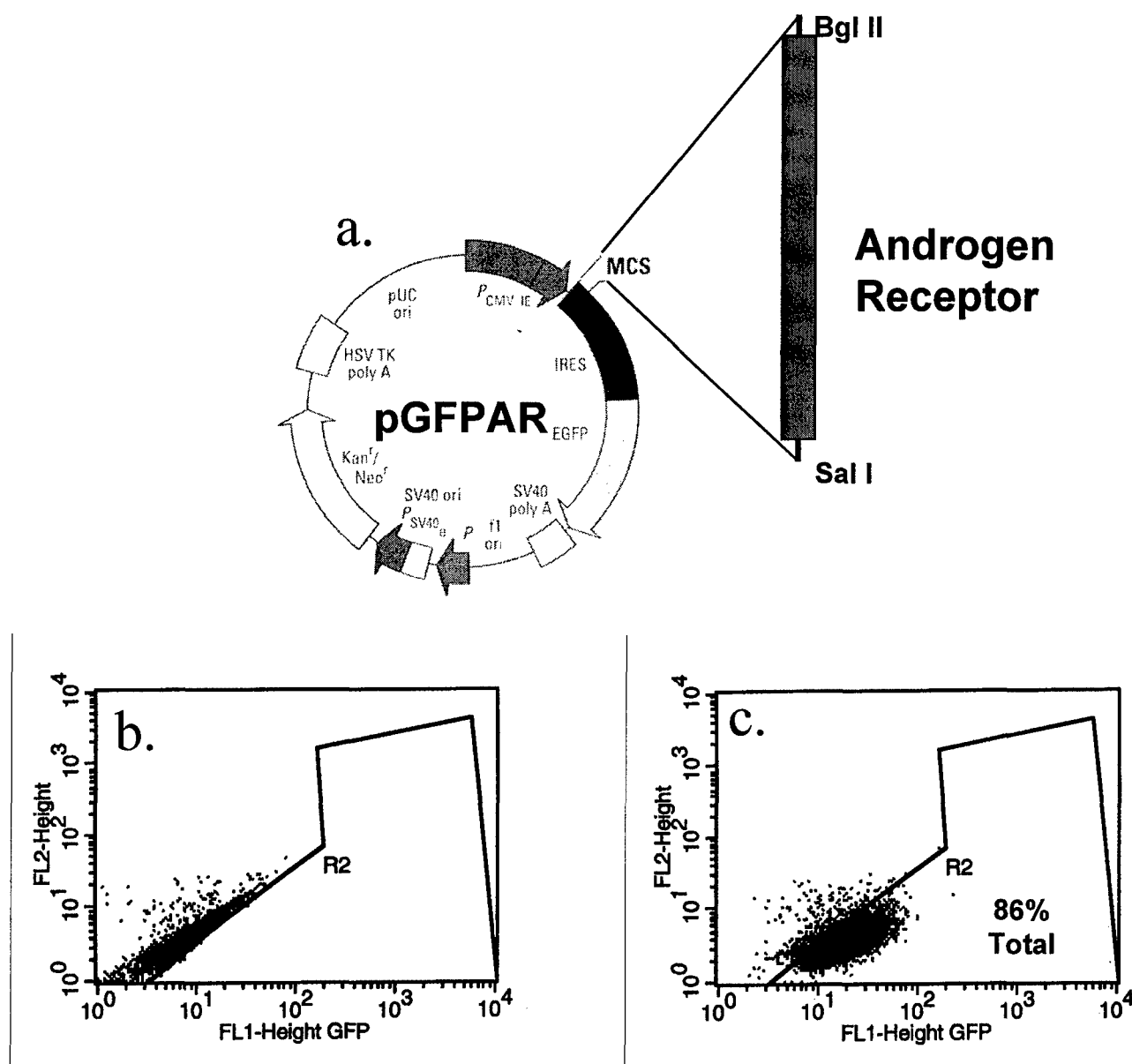
Appendix 2, Figure 18

Figure 18. pGFPAR expression vector construct and expression in PC3 cells. (a) pGFPAR was constructed as an expression vector to allow the screenable transduction of the human androgen receptor to human prostate cells. The human androgen receptor was subcloned from the phCMVAR construct (kindly provided by Dr. Wayne Tilley) in to the BglI and SalI sites of the multiple cloning sequence (MCS). (b) Flow cytometric analysis of enhanced green fluorescence protein (EGFP) expression in PC3 cells gated to exclude EGFP positive cells. (c) Cells stably transduced with pGFPAR were found efficiently express EGFP, with up to 86% positive cells.
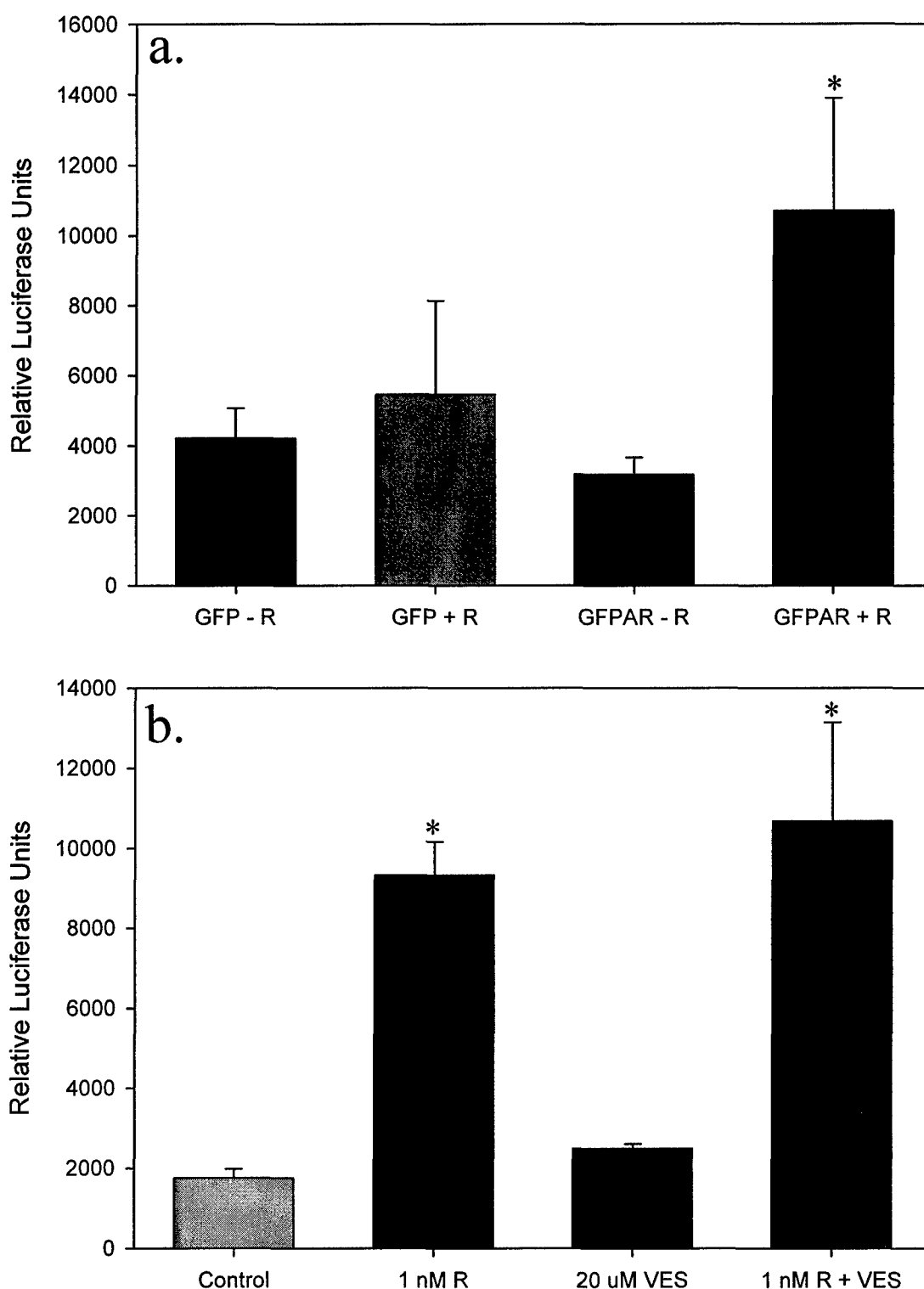
Appendix 2, Figure 19

Figure 19. Androgen activation of an androgen-responsive promoter in PC3 human prostate carcinoma cells expressing pGFPAR with and without vitamin E succinate treatment. (a) PC3 cells were cotranfected with the control pIRES2.EGFP vector (GFP) and an androgen-responsive MMTV-luciferase plasmid (pMMTVlux) or with pGFPAR and MMTVlux using Fugene 6 and treated with or without 1 nM R1881. (b) PC3 cells cotransfected with pGFPAR and MMTVlux and treated with 1 nM R1881, 20 μM vitamin E succinate, or 1 nM R1881 + 20 μM vitamin E succinate. *$P<0.05$ compared to control.
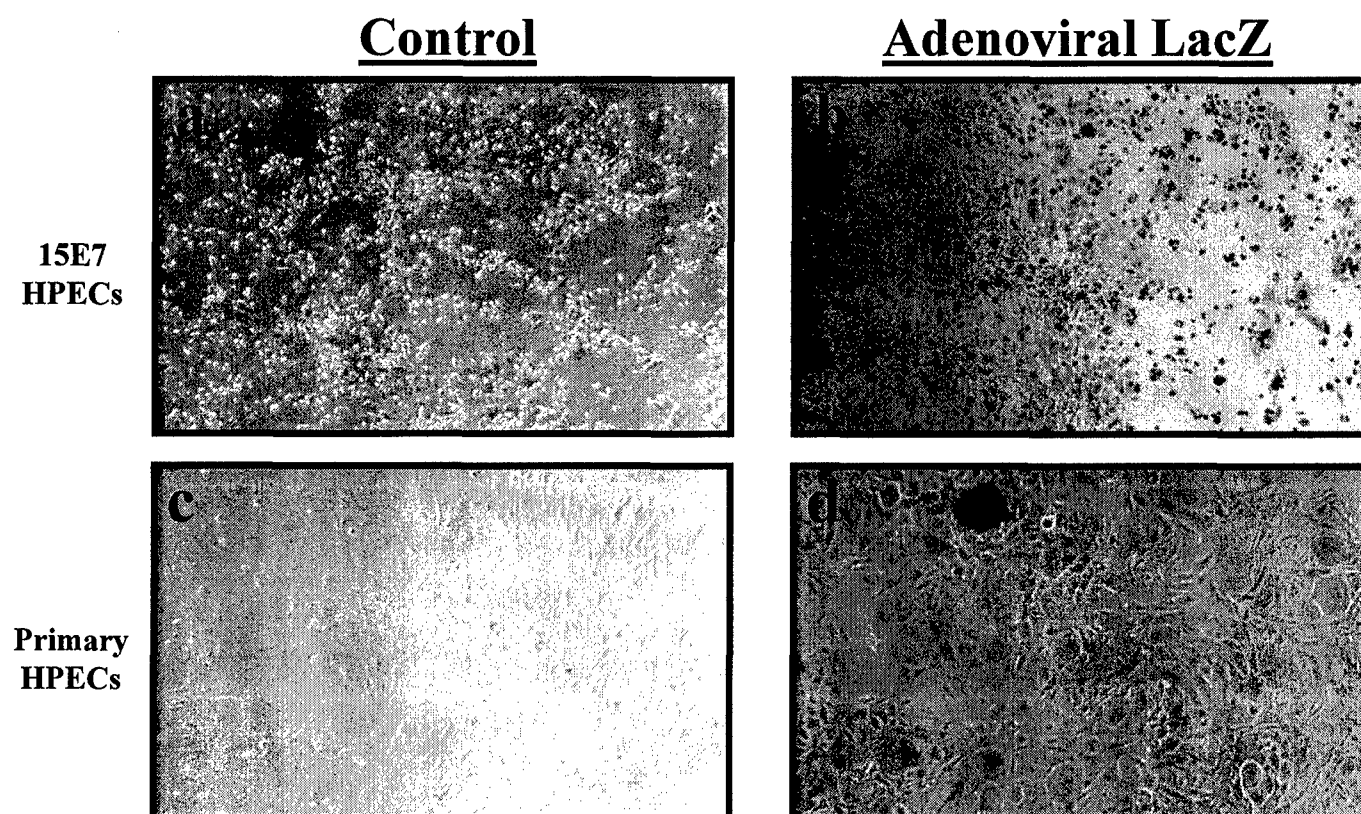
Appendix 2, Figure 20

Figure 20. Adenoviral infection of human prostate epithelial cells (HPECs). Immortalized 15E7 HPECs (a, b) were acquired from Dr. David Jarrard's laboratory (U.W. Dept. of Surgery). HPECs were treated as control uninfected 15E7 cells (a) or as adenoviral-infected 15E7 cells (b), at an MOI of 100/cell, with a vector expressing LacZ and were stained with X-gal. Similarly, primary human prostate epithelial cells (c, d) were acquired from Dr. David Jarrard's laboratory and infected with an adenoviral vector expressing LacZ (d) and stained with X-gal. For both immortalized and primary prostate cells, adenoviral infection efficiency was greater than 95%. Background X-gal staining was not observed in uninfected cells (a, c).

Appendix 3

Church DR, Lee E, Thompson TA, Basu HS, Ripple MO, Ariazi EA, Wilding G. Induction of AP-1 activity by androgen activation of the androgen receptor in LNCaP human prostate carcinoma cells. The Prostate (published online in Wiley InterScience, December 2004; in press)

Induction of AP-1 Activity by Androgen Activation of the Androgen Receptor in LNCaP Human Prostate Carcinoma Cells

Dawn R. Church,[1] Elyse Lee,[1] Todd A. Thompson,[1] Hirak S. Basu,[1]
Maureen O. Ripple,[1] Eric A. Ariazi,[2] and George Wilding[3]*

[1]University of Wisconsin Comprehensive Cancer Center, University of Wisconsin, Madison
[2]McArdle Laboratory for Cancer Research, University of Wisconsin, Madison
[3]University of Wisconsin Comprehensive Cancer Center and Department of Medicine, University of Wisconsin, Madison

BACKGROUND. The androgen receptor and activator protein-1 (AP-1) transcription factors affect growth regulation in normal and cancerous prostate cells. Effects of androgen-activated androgen receptor on AP-1 activity were determined in the LNCaP human prostate carcinoma cell model.

METHODS. Cells were exposed to 1 nM androgen ± antiandrogen bicalutamide. Cellular growth and cell cycle effects were determined by DNA, viability, and bromodeoxyuridine (BrdU) fluorescence activated cell sorter (FACS) assays. AP-1 effects were determined by an AP-1-luciferase enzyme reporter vector for transcriptional activity, electrophoretic mobility shift assay (EMSA)/antibody supershift for DNA-binding, quantitative RT-PCR for mRNA, and immunoblot for protein.

RESULTS. Androgen induced G_1 growth arrest. This growth arrest was abrogated by treatment with bicalutamide, demonstrating that growth arrest by androgen was due to androgen receptor activation. Concurrently, AP-1 DNA-binding and transcriptional activity was induced over 96 hr androgen exposure, which was also inhibited by bicalutamide. Interestingly, although no change in AP-1 transcriptional activity was observed 24 hr after androgen exposure, there was an increase in Fra-2 expression and AP-1 DNA-binding. Paradoxically, while Fra-2 mRNA and protein levels continued to increase, binding of Fra-2 to the AP-1 site decreased over 96 hr, with a concomitant increase in JunD AP-1-binding and a marked increase in expression of the 35 kDa form of JunD. Enhanced expression of this short form of JunD is a novel effect of androgen exposure that occurred during the 24–96 hr time period, as growth effects emerged.

CONCLUSION. Activation of androgen receptor by androgen induces changes in AP-1 activity and AP-1 factor DNA-binding that may contribute significantly to androgen-induced changes in prostate cancer cell growth. © 2004 Wiley-Liss, Inc.

KEY WORDS: cellular growth arrest; Fra-2; JunD

INTRODUCTION

The central role of androgens in the regulation of growth, differentiation, and death responses in both normal and cancerous prostate tissue is well recognized [1–4]. However, the molecular signaling pathways induced by androgen exposure are not clearly defined. Androgens predominantly exert their effects through the ligand-activated androgen receptor [5,6]. The activated androgen receptor initiates a cascade of signaling events that result in diverse cellular changes. The importance of defining the signaling pathways induced by activation of the androgen receptor is underscored by the evidence that even in the advanced, androgen-independent disease state of prostate cancer, androgen receptor signaling pathways may be functioning and have a role in the progression of the disease [6]. An understanding of these pathways is critical to understanding the biology of prostate homeostasis versus prostate cancer and may help identify molecular targets for prevention/treatment of prostate cancer.

Activator protein-1 (AP-1) transcription factors comprise a ubiquitously expressed family of proteins that include the Jun (e.g., cJun, JunB, JunD) and Fos (e.g., cFos, FosB, Fra-1, Fra-2) proto-oncoproteins [7]. The AP-1–DNA-binding complex is composed of Jun–Jun or Jun–Fos dimers which bind to the AP-1 consensus sequence TGA(C/G)TCA [7]. The AP-1 complex is a "signal converter" [8], which mediates responses to cellular signals by binding DNA and producing changes in gene transcription that ultimately lead to physiologic changes in the cell. AP-1 activity is implicated in many different cellular processes including cell proliferation, differentiation, apoptosis, and stress responses. The cellular response to AP-1 binding depends on the composition of the AP-1 complex, the target genes that are activated and the cell type [7,9,10]. The relevance of AP-1 for human diseases is not completely understood, but it is thought that AP-1 proteins may be important factors in the pathogenesis of human cancers, since the AP-1 complex is a conversion point of many signaling pathways [11]. A recent report from Zerbini et al. [12] showed that activation of AP-1 factors in human prostate cancer cells contributes to deregulation of interleukin-6, which is associated with advanced, androgen-independent human prostate cancer. This suggests that AP-1 activity may be important in prostate cancer development/progression.

The cellular changes induced by activation of the androgen receptor may occur directly by modulation of transcriptional activity mediated by the androgen receptor binding to androgen response elements. In addition, cellular changes following androgen exposure can occur through indirect means. For example, we have previously shown that androgen exposure in the forms of 5α-dihydrotestosterone or the synthetic androgen R1881 can result in changes in the cellular reduction/oxidation (redox) state in androgen-responsive human prostate cancer cells [13]. These androgen-mediated changes may affect transcription factors that are sensitive to cellular redox status, such as the AP-1 proteins [14,15]. Additionally, both transrepressive and transactivating cross-talk can occur between the androgen receptor and AP-1 [16,17]. Thus, AP-1 activity may be directly or indirectly affected by androgen exposure. Changes in the time-course and specificity of AP-1 factor activity may be important in the androgen response of prostate cells in general and prostate cancer cells in particular.

Androgens can induce growth stimulatory signaling pathways for proliferation of prostate cells as well as pathways associated with growth inhibition such as differentiation of prostate cells. The LNCaP human prostate carcinoma cell line provides a useful model for studying androgen receptor signaling pathways in relation to growth regulation of prostate cancer cells, because LNCaP cells exhibit a robust biphasic growth stimulation–growth inhibition response to a near physiologic androgen concentration range [18]. In previous studies, we demonstrated that exposure to androgens 5α-dihydrotestosterone or R1881 at 1 nM levels, which is similar to human plasma androgen levels [19], induces growth inhibition in the LNCaP model [13]. In further studies of androgen signaling in this model, we identified that growth inhibitory levels, but not growth stimulatory levels, of androgen cause oxidative stress and a prolonged increase in AP-1 DNA-binding activity [13,20]. Lack of effect on growth, reduction/oxidation state, or AP-1 DNA-binding activity by androgen exposure in DU145 human prostate carcinoma cells [13,20], which do not express functional androgen receptor [21], suggested that these effects are likely mediated by androgen signaling through the androgen receptor. Others have shown that growth inhibitory concentrations of androgens can produce an increase in $p27^{Kip1}$ protein resulting in an inhibition of cyclin-dependent kinase 2 activity and G_1 cell cycle arrest in an LNCaP model [22]. Together, these studies suggest that activation of the androgen receptor by androgen can trigger a cellular growth arrest response that involves AP-1 signaling activity.

This study was performed to investigate the effects of androgen on the activity of AP-1 in LNCaP cells in relation to activation of the androgen receptor. All studies were carried out over 24 or 96 hr of androgen exposure to complement data from previous studies that demonstrated a time course of androgen effects on cellular growth and AP-1 DNA-binding activities. In previous studies, androgen-induced changes in AP-1 DNA-binding were observed prior to changes in

growth, and were maximal at 96 hr after androgen exposure [13,20]. The androgen effects on growth were not measurable before 2–3 days after androgen exposure [13,20]. Since cellular responses mediated by AP-1 are dependent on the complex interplay between the different dimers of the Jun and Fos family members, the specific changes in AP-1 DNA binding factors induced by androgen exposure were determined. JunD and Fra-2 were identified as key components of the AP-1 complex that underwent changes as AP-1 DNA-binding and transcriptional activity increased in temporal association with androgen-induced growth inhibition of LNCaP cells. An attenuation of the androgen effects on growth inhibition and AP-1 by antiandrogen bicalutamide shows that these effects are dependent on activation of the androgen receptor, and suggests a role for AP-1 activation in androgen-androgen receptor signaling for growth inhibition.

MATERIALS AND METHODS

Cell Culture

The androgen-responsive LNCaP and androgen-independent DU145 human prostate carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C under humidified air containing 5% CO_2 in Dulbecco's MEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 5% heat-inactivated FBS (Invitrogen Life Technologies) and 1% of 100× antibiotic, antimycotic solution (Sigma Chemical Co., St. Louis, MO). Cells were passed weekly and medium was replaced 3 or 4 days after passing. LNCaP cells of passage 50–90 were used for all experiments to maintain consistency in cell responses to androgen. Cells for experiments were seeded in medium containing 4% charcoal-stripped FBS plus 1% non-stripped FBS, designated F1C4 medium. This combination of stripped and non-stripped serum was previously shown to sufficiently deplete androgen content while limiting adverse growth effects not related to hormone depletion [13].

Cellular Growth and Viability Analysis

For determination of growth response to androgen, LNCaP cells were seeded in 96-well tissue culture plates at a density of 2,500 cells per well in 100 µl of F1C4 medium. Treatments were administered three days after seeding by adding an equal volume of medium (i.e., 100 µl) containing either 2 nM of the synthetic androgen R1881 (methyltrienolone, metribolone; DuPont, NEN, Boston, MA), 10 µM bicalutamide (Casodex, ICI17,334; gift from AstraZeneca Pharmaceuticals, Wilmington, DE), 2 nM R1881 + 10 µM bicalutamide, or 0.02% ethanol to yield final treatment concentrations of 1 nM R1881,

5 µM bicalutamide, 1 nM R1881 + 5 µM bicalutamide, and 0.01% ethanol (i.e., vehicle control). At 24 or 96 hr after R1881 exposure, cultures were harvested by washing and incubation in Kreb's Ringer buffer for 1 hr prior to freezing at −80°C. Cell growth was determined by measuring cellular DNA levels as previously described [23]. Cell viability was determined by trypan blue exclusion and quantified by light microscopic analysis using a hemacytometer.

Cell Cycle Analysis

For analysis of cell cycle distribution LNCaP cells were seeded in 6-well plates at a density of 1×10^5 cells/well in 1.5 ml F1C4 medium and treated as described for cellular growth, adding treatment volumes of 1.5 ml/well. At 96 hr after R1881 exposure, cells were incubated for 1 or 4 hr with 20 µM bromodeoxyuridine (BrdU; Sigma) for labeling of S-phase cells, then collected by trypsinization, washed with PBS, and fixed by drop-wise addition of 95% ethanol. Nuclei were collected by disruption with 0.04% pepsin (Sigma) in HCl and incubated with an anti-BrdU antibody (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) followed by an anti-mouse IgG-fluorescein isothiocyanate-conjugated (FITC) antibody (Sigma). Nuclei were further stained with propidium iodide (Sigma) at 50 µg/ml in the presence of 50 µg/ml DNAse-free RNAse A (Sigma) overnight at 4°C. Cell cycle distribution was analyzed using a Becton Dickinson FACScan flow cytometer, collecting 30,000 events per sample. Events were gated for G_1, S, and G_2/M and normalized to 100% events.

AP-1 Transcriptional Activity Analysis

AP-1 and mutant AP-1 (mAP-1) sense and antisense oligonucleotides containing five AP-1 DNA consensus sites were synthesized by Sigma GenoSys (Woodlands, TX). AP-1(5×) and mAP-1(5×) oligonucleotides were PCR amplified with primers containing XhoI and BglII restriction sites synthesized by the University of Wisconsin-Biotechnology Center (Madison, WI). AP-1(5×) primer sequences are 5′-GAC TCT CGA GTC GAG TGA TGA GTC AGC CGG A-3′ and 5′-GTA CAG ATC TTC GGC TGA CTC ATC ACG CAT-3′. mAP-1(5×) primer sequences are 5′-GAC TCT CGA GTC GAG TGA CGT GTG AGC CGG A-3′ and 5′-GTA CAG ATC TTC GGC TCA CAC GTC ACG CAT-3′. The 116 bp PCR product was purified by gel electrophoresis, codigested with XhoI/BglII enzymes, and ligated into the corresponding insertion sites in the pGL3-basic firefly luciferase enzyme reporter vector (Promega, Madison, WI). AP-1(5×)luc and mAP-1(5×)luc plasmids were sequenced to confirm correct AP-1(5×) fragment insertion.

LNCaP cells were cultured in opaque, clear bottom 96-well plates at a density of 6,000 cells/well and incubated in F1C4 media for 24 hr, resulting in 50–70% confluence. Cells were transiently transfected with 0.1 μg of AP-1(5×)luc, mAP-1(5×)luc, or empty (i.e., promotorless) plasmid using the Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. To normalize for transfection efficiency, cells were co-transfected with 0.1 μg of the TK-βgal β-galactosidase reporter vector (Clontech, Palo Alto, CA). After 3 hr of transfection, media was removed from cells and replaced with F1C4 media containing 1 nM R1881 (DuPont, NEN), 1 μM bicalutamide (AstraZenenca), 1 nM R1881 + 1 μM bicalutamide, or 0.01% ethanol (i.e., vehicle control). Cells were harvested 24 and 96 hr after R1881 exposure in 30 μl of luciferase extraction buffer [0.1 M KH$_2$PO$_4$ (pH 7.5), 1 mg BSA, 400 μM phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol]. Cells were lysed by one freeze/thaw cycle.

For analysis of β-galactosidase expression, 10 μl of extract was incubated with 3 μg of the β-galactosidase substrate 4-methylumbelliferylgalactopyranoside (MUG; Molecular Probes, Eugene, OR) in sample buffer (600 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO, 10 mM KCl, 1 mM MgSO$_4$, pH 7.0). The reaction was incubated for 1 hr at room temperature and terminated with stop buffer (300 mM glycine, 15 mM EDTA, pH 11.2). MUG fluoresecence was quantified using a SPECTRAmax GEMINI dual scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) with excitation at 350 nm and emission at 450 nm.

For the luciferase enzyme assay, 20 μl of cell extract was incubated with 80 μl of reaction buffer (30 mM MgCl$_2$, 30 mM glycylglycine, 4 mg/ml BSA, 10 mM ATP, pH 7.8). Ten microliters of luciferin (Molecular Probes) solution (0.4 mg/ml in 10 mM NaHCO$_3$, pH 6.0) was injected into each sample well and measured for 1 sec for luciferin light emission using the TROPIX TR717 Microplate Luminometer (Applied Biosystems).

Luciferase enzyme activity (i.e., relative light units, RLU) was normalized to β-galactosidase activity (i.e., relative fluorescence units, RFU) for each sample. The fold differences between samples from cells exposed to R1881, bicalutamide, or R1881 plus bicalutimide compared to vehicle control were calculated using mean RLU/RFU values and associated error.

EMSA /Antibody Supershift Assays

For protein analysis, LNCaP cells were seeded at a density of 1 × 10^6 cells and DU145 cells at 5 × 10^5 cells in 8 ml of F1C4 medium per 100 mm tissue culture plate. Cells were exposed to androgen 3 days after seeding. Medium was replaced with 8 ml of fresh treatment medium containing either 1 nM R1881 (DuPont, NEN), 5 μM bicalutamide (AstraZeneca Pharmaceuticals), 1 nM R1881 + 5 μM bicalutamide, or 0.01% ethanol (i.e., vehicle control). Cultures were harvested 24 or 96 hr after R1881 exposure. Cultures were placed on ice and washed once with cold Kreb's Ringer buffer. Cells were collected by scraping and resuspended in cold Kreb's Ringer buffer containing freshly added 0.2 mM PMSF (Sigma), then pelleted by centrifugation at 1,000 rpm for 5 min at 4°C. Cell pellets were snap frozen in liquid nitrogen and stored at −80°C until extraction of nuclear proteins according to the method of Andrews and Faller [24] with some modification as previously described [20]. Protein content of extracts was measured using the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL) and extracts were snap frozen and stored at −80°C.

Five micrograms of nuclear protein extracts were analyzed for AP-1 DNA-binding activity using the method of Andrews and Faller [24] with some modification as previously described [20]. For supershifts, antibodies were added at 1 μl each to the reaction mix simultaneous with AP-1 oligodeoxynucleotide (Promega) radiolabeled with ^{32}P. Antibodies used included pan cFos(K-25) (SC-253×), cFos(4) (SC-52×), FosB(102) (SC-48×), Fra-1(R-20) (SC-605×), Fra-2(Q-20) (SC-604×), JunD(329) (SC-74×), cJun(H-79) (SC-1694×), JunB(N-17) (SC-46×), and JAB1(FL-334) (SC-9074×), all of which were TransCruz gel shift rabbit polyclonal antibodies concentrated at 2 mg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). Two micrograms per 1 μl were used in the assay. The binding reaction mix was incubated for 20 min at room temperature. Specificity of AP-1 DNA-binding activity was shown in each assay by incubation of radiolabeled AP-1 oligodeoxynucleotide with water only (no protein) and by incubation of a sample with 1 μl (1.75 pmol) of non-radiolabeled AP-1 or OCT-1 oligodeoxynucleotide competitors (Promega). DNA–protein–antibody/non-radiolabeled competitor complexes were electrophoresed through a 1.5 mm, 4.5% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer at 175 V for approximately 2 hr. Gels were dried onto Whatman 3M paper using a Gel Dryer Model 583 (BioRad Laboratories, Hercules, CA) and imaged after 18 hr of exposure on a Storage Phosphor Screen. Gels were scanned using a Storm860 system and images were analyzed using ImageQuant analysis software (Amersham Biosciences, Piscataway, NJ).

Analysis of mRNA Abundance by Quantitative Real-Time RT-PCR (qRT-PCR)

LNCaP cultures for RNA analysis were set-up as described for EMSA and harvested using the Total

RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA samples were snap frozen and stored at −80°C. The mRNA abundances of JunD and Fra-2 were measured by SYBR green I-based qRT-PCR assays. To control for variability in the amount of RNA and cDNA synthesis efficiency between samples, mRNA levels of the genes under investigation were normalized to the mRNA levels of the acidic ribosomal phosphoprotein *P0* gene, identified as the *36B4* gene.

Total RNA samples were pre-treated with RNase-free DNAseI (Ambion, Austin, TX) and RNA was re-isolated using the Total RNeasy kit (Qaigen) according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using SuperScript II reverse transcriptase (Invitrogen Life Technologies) and 10 nmoles each of oligo $dT_{15}VN$ (where V = A, G, or C and N = any nucleotide) and random hexamers as primers. SYBR Green I (Molecular Probes) was diluted in anhydrous DMSO at 1:2,500, then added to the enzyme reaction buffer to obtain a final concentration of 1:50,000 SYBR green I and 5% DMSO. To normalize fluorescence intensity between samples, the enzyme reaction buffer contained 180 nM passive reference dye ROX (Molecular Probes). The thermal cycling parameters were 1 cycle of 95°C for 10 min; and 40 cycles of 96°C denaturation for 15 sec followed by 60°C annealing/extension for 1 min. The PCR primer set sequences and amplicon sizes were as follows:

JunD (70 bp amplicon)	Forward primer 5'-CTC GCG CCT GGA AGA GAA A-3'
	Reverse primer 5'-CAG GCT CGC CGT GGA C-3'
Fra-2 (79 bp amplicon)	Forward primer 5'-GAT CAA GAC CAT TGG CAC CAC-3'
	Reverse primer 5'-GCG ACG CTT CTC CTC CTC T-3'
36B4 (95 bp amplicon)	Forward primer 5'-CCT CAT ATC CGG GGG AAT GTG-3'
	Reverse primer 5'-GCA GCA GCT GGC ACC TTA TTG-3'

PCR primer sequences were designed using Oligo 5.0 software (National Biosciences; Plymouth, MN) and synthesized at the University of Wisconsin-Biotechnology Center (Madison, WI). The qRT-PCR assays were carried using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Construction of serial dilution standard curves of each specific PCR product were included in every experiment and allowed calculation of transcript copy numbers by regression analysis. The concentrations of PCR products for the standard curves were determined by spectrophotometric analysis and converted into copy numbers according to the molecular weight of the specific amplicon's size in base pairs. The standard curves covered at least 7 orders of magnitude with each known concentration assayed in triplicate for a total of 21 measurements. The mean copy number of triplicate measurements for a particular mRNA species was normalized to the mean of quadruplicate measurements of 36B4's mRNA copy number within every sample. The mean target to 36B4 mRNA molar ratio for each sample was used in determining the average copy number and associated standard deviation for each treatment condition.

Immunoblots

LNCaP cell proteins were prepared as described for EMSA. Sodium dodecyl sulfate–PAGE was performed according to the methods described by Bollag et al. [25]. Twenty-five micrograms of nuclear protein extracts were brought to equal volumes for loading. Samples were heat denatured at 95°C for 5 min and placed on ice for at least 2 min prior to loading on sodium dodecyl sulfate–12% polyacrylamide Tris-HCl Ready Gels (BioRad) for electrophoresis. Kaleidoscope Prestained Standards (BioRad) were used for determining relative migration molecular weight. Proteins were transferred from the gel onto Immobilon-P membranes (Millipore Corp., Bedford, MA) using the GENIE wet transfer system (Idea Scientific, Minneapolis, MN). After transfer, gels were stained with GELCODE Blue Stain Reagent (Pierce) per the manufacturer's instructions and observed for evidence of equal loading and protein transfer. Membranes were blocked in tris-buffered saline containing 5% powdered milk at 4°C overnight and then incubated with primary antibody. Primary antibodies used included the JunD(329) (SC-74×) and Fra-2(Q-20) (SC-604×) antibodies. Membranes were then incubated with a horseradish peroxidase-labeled secondary rabbit antibody (NIF 824) (Amersham Biosciences) and analyzed using Enhanced Chemiluminescence Plus reagent (Amersham Biosciences). Autoradiograms were prepared by exposing immunoblots to Biomax X-ray film (Eastman Kodak Company, Rochester, NY) and developed on a CURIX 60 CP Processor (Agfa, Ridgefield Park, NJ).

Statistical Analysis

Cellular growth analysis was performed in three experiments and included six samples per treatment condition within each experiment. Cell cycle analysis was performed in two experiments, each including three samples per treatment condition. Transcriptional activity analysis experiments were performed three times with at least three individual samples per

treatment condition within each experiment. Experiments for mRNA analysis were performed twice and included three individual RNA samples per treatment condition within each experiment. Protein analysis experiments were performed at least two times including two or three individual protein samples per treatment condition within each experiment. Treatment conditions were compared using an unpaired two-tailed heteroscedastic Student's t-test with a confidence level of 0.05. Significant P values are reported.

RESULTS

Androgen-Induced Growth Arrest in LNCaP Cells

The growth of LNCaP cells was determined at 24 and 96 hr after exposure to 1 nM R1881. At 24 hr after exposure, no difference in cell growth as measured by DNA content determination was found between cells exposed to 1 nM R1881 or vehicle control medium (Fig. 1A). By 96 hr, however, the growth of LNCaP cells exposed to 1 nM R1881 was 64% that of control cells (Fig. 1A). No change in cell viability as measured by trypan blue exclusion was observed for cells exposed to 1 nM R1881 at either 24 or 96 hr compared to vehicle-treated controls (data not shown). Cell cycle analysis was performed on cells exposed to androgen for 96 hr to determine the stage of growth inhibition. The results are shown in Figure 1B and Table I. Cells exposed to 1 nM R1881 for 96 hr were 7% more in G_1 phase, 5% less in S phase, and 1% less in G_2/M phase compared to control cells (Table I). To determine the role of androgen receptor activation on the growth inhibition observed after 96 hr of androgen exposure, LNCaP cells were additionally treated with 5 µM of the antiandrogen bicalutamide, which specifically competes with androgen for binding to the androgen receptor [26]. Co-administration of bicalutamide attenuated the growth inhibition (Fig. 1A) and G_1 arrest (Fig. 1B and Table I) induced by 1 nM R1881.

Androgen-Induced AP-I Transcriptional Activity in LNCaP Cells

The effect of androgen exposure on AP-1 transcriptional activity in LNCaP cells was measured using an AP-1-driven luciferase enzyme reporter vector. There was no difference in AP-1 transcriptional activity between LNCaP cells exposed to vehicle or 1 nM R1881 after 24 hr (Fig. 2). After 96 hr androgen exposure, however, a 5-fold increase in AP-1 transcriptional activity was observed in these cells (Fig. 2). The induction of AP-1 transcriptional activity was blocked nearly 50% by co-treatment with 1 µM bicalutamide (Fig. 2). Specificity of AP-1 activation was determined using a luciferase enzyme reporter vector driven by mAP-1 sites or a promoterless luciferase expression vector as negative controls: no difference in luciferase expression was observed between LNCaP cells exposed to vehicle or 1 nM R1881 for 24 or 96 hr after transfection with either the mutant vector or the promoter less vector controls (data not shown).

Characterization of AP-I DNA-Binding Complex Proteins Following Androgen Exposure in LNCaP Cells

The binding activity and composition of the AP-1 DNA-binding complex in LNCaP cells were analyzed by electrophoretic mobility shift assay (EMSA) and antibody supershift assays using nuclear protein extracts and anti-Fos or anti-Jun antibodies. LNCaP cells exposed to 1 nM R1881 exhibited a relative increase in DNA-binding activity to the AP-1 consensus sequence at both 24 and 96 hr after exposure as compared to vehicle-treated control cells (Fig. 3). This effect was attenuated by co-treatment with 5 µM bicalutamide (Fig. 3). Initial gel supershift studies were performed with a panFos antibody that recognizes multiple Fos family members. A Fos member was detected in the complex in control cells and in cells exposed to 1 nM R1881 for 24 hr. However, no Fos supershift was observed in cells exposed to 1 nM R1881 for 96 hr. Further studies were performed with antibodies to individual Fos members to identify specific Fos family members involved in the AP-1 binding. Similar to the gel supershift results for the panFos antibody, Fra-2 was detected in the AP-1 DNA-binding complex using an anti-Fra-2 antibody at both 24 and 96 hr for vehicle-treated cells, whereas Fra-2 was detected in the supershifted AP-1 complex at 24 hr, but was markedly reduced at 96 hr for cells exposed to 1 nM R1881 (Fig. 4A,B). Antibodies for cFos, FosB, and Fra-1 did not cause a shift or reduction of the AP-1 binding complex band in cell extracts at either 24 or 96 hr (Fig. 4A,B). Utility of these antibodies for supershift analysis was demonstrated using nuclear extracts from DU145 cells as described below. JunD was detected in the AP-1 DNA-binding complex at both 24 and 96 hr in cells exposed to either 1 nM R1881 or vehicle using an anti-JunD antibody (Fig. 5A,B). Antibodies for cJun, JunB, and the transcriptional co-activator JAB1 did not cause a shift or reduction of the AP-1 binding complex band in extracts from cells treated for either 24 or 96 hr (Fig. 5A,B).

To further investigate the relevance of androgen receptor activation to the effects observed in AP-1 DNA binding activity and the specific AP-1 factors induced by androgen exposure, similar studies were performed in androgen-insensitive DU145 human prostate carcinoma cells, which do not express functional androgen
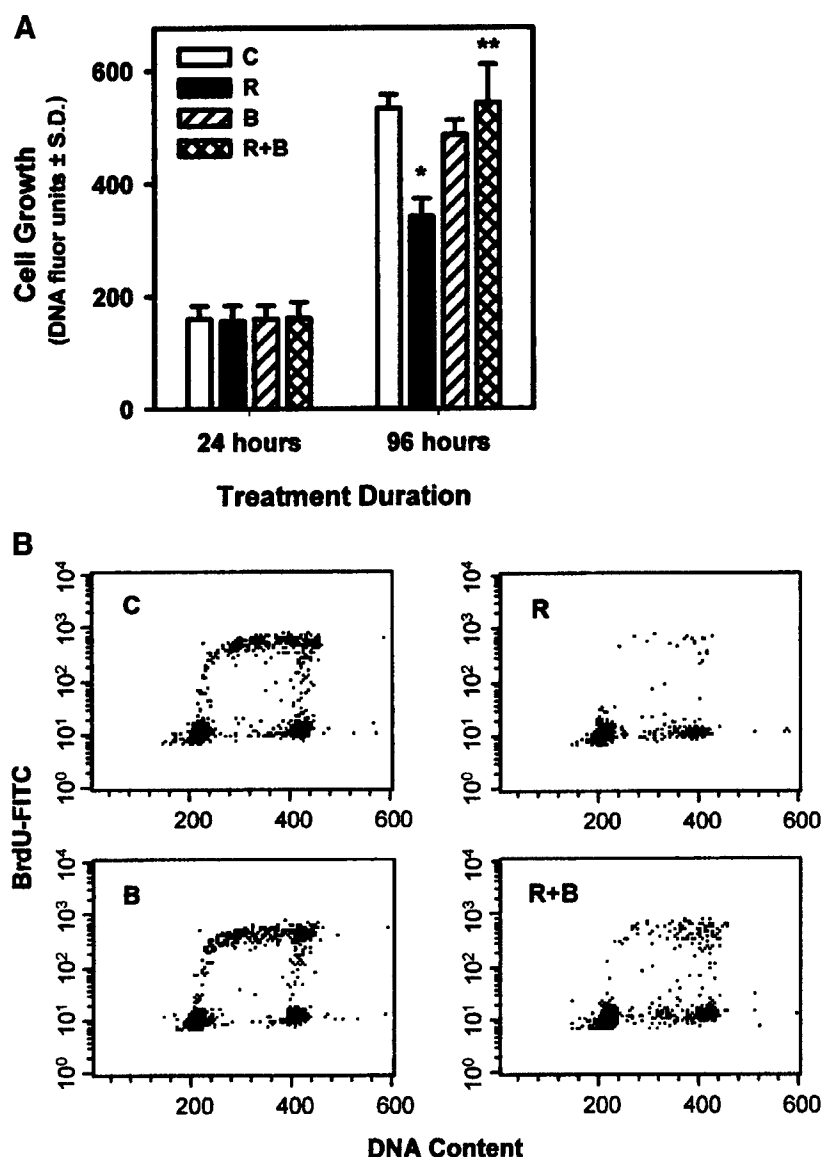
Fig. I. LNCaP cell number determination at 24 and 96 hr (**A**) and cell cycle phase distribution at 96 hr (**B**) after androgen RI881 exposure ± antiandrogen bicalutamide. **A**: LNCaP cells exposed to vehicle control medium (C, open bars), I nM of the synthetic androgen RI881 (R, solid bars), 5 µM of antiandrogen bicalutamide (B, hatched bars), or the combination of I nM RI881 and 5 µM bicalutamide (R + B, crossed bars) were analyzed for DNA levels as an indicator of growth at 24 and 96 hr after exposure. At each timepoint, levels of DNA were measured using Hoechst fluorescent dye and expressed as DNA fluor units. DNA fluor units represents cellular growth because the same number of cells were plated per well. By 96 hr, growth of LNCaP cells exposed to I nM RI881 was 64% that of control LNCaP cells treated with vehicle only (*$P < 0.0001$, compared to control). The growth inhibition due to I nM RI881 exposure was effectively blocked by the addition of 5 µM bicalutamide (**$P < 0.0001$, compared to I nM RI881). Each data point is the average ± standard deviation of six independent samples. Studies were performed three times. **B**: At 96 hr, LNCaP cells that were treated as above were incubated with bromodeoxyuridine (BrdU) for 4 hr prior to trypsinization and harvest of nuclei. Thirty-thousand nuclei per sample were analyzed by fluorescence activated cell sorter (FACS) analysis for determination of cell cycle distribution. G_1, S, and G_2/M phases were gated based on the BrdU–fluorescein isothiocyanate-conjugated (FITC) (anti-BrdU–anti-IgG-FITC antibody staining) versus DNA content (propidium iodide staining) plots. The percent phase distribution is summarized in Table I. LNCaP cells exposed to I nM RI881 were arrested in the G_1 phase compared to control cells treated with vehicle only (*$P < 0.05$, compared to control), and the addition of 5 µM bicalutamide to I nM RI881 attenuated this cell cycle arrest (**$P < 0.05$, compared to I nM RI881). N = 3 individual samples analyzed by FACS per condition. Data were confirmed by a second experiment in which a I hr BrdU labeling was performed.

TABLE I. Cell Cycle Distribution for LNCaP Cells at 96 hr After Exposure to Androgen

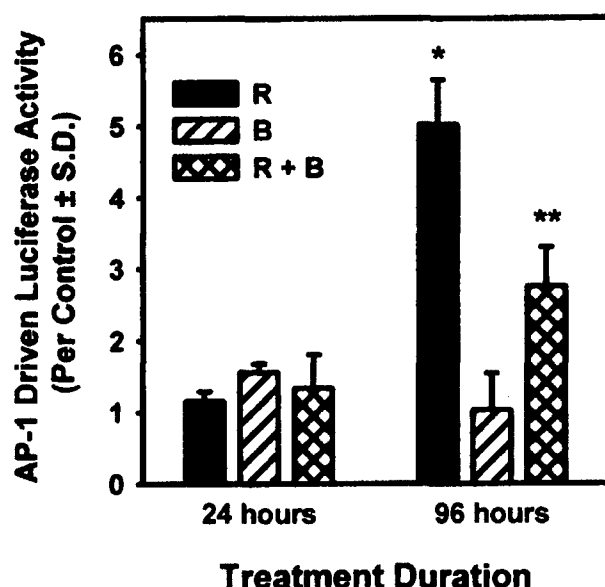| | % Cells in Phase ± SD | | |
| | | Cell Cycle Phase | |
Treatment (n = 3)	G_1	S	G_2/M
Control	90.0 ± 0.2	5.2 ± 0.3	4.8 ± 0.4
1 nM R1881	96.6 ± 0.7*	0 5 ± 0.1*	2.9 ± 0.7*
5 μM bicalutamide	90.2 ± 1.1**	6.1 ± 0.3*·**	3.7 ± 0.9
1 nM R1881 + 5 μM bicalutamide	93.5 ± 0.4*·**	2.5 ± 0.2*·**	4.0 ± 0.5

*$P < 0.05$ compared to control treatment.
**$P < 0.05$ compared to 1 nM R1881 treatment.

Fig. 2. Effect of R1881 exposure on activator protein-1 (AP-1) transcriptional activity in LNCaP cells at 24 and 96 hr after exposure. LNCaP cells were transiently co-transfected with an AP-1 (5×) luciferase enzyme reporter vector and a β-galactosidase reporter vector containing a minimal thymidine kinase promoter for 3 hr, then exposed to I nM R1881 (R, solid bars), I μM antiandrogen bicalutamide (B, hatched bars), I nM R1881 +I μM bicalutamide (R + B, crossed bars), or vehicle control medium (normalization factor, per control). Extracts from cells harvested at 24 or 96 hr after exposure were analyzed for luciferase activity and β-galactosidase activity. Luciferase activity was normalized to β-galactosidase activity for each of three individual samples per treatment condition at each timepoint. Values for R, B, and R + B samples were normalized per the ratio of luciferase activity to β-galactosidase activity for control cells treated with vehicle only at the respective timepoint (per control ± SD). Exposure to I nM R1881 produced a 5-fold increase in AP-1 transcriptional activity by 96 hr in LNCaP cells (*$P = 0.004$, compared to control). One micromolar bicalutamide inhibited the R1881 induced AP-1 transcriptional activity by nearly 50% (**$P = 0.03$, compared to I nM R1881). Studies were performed three times.
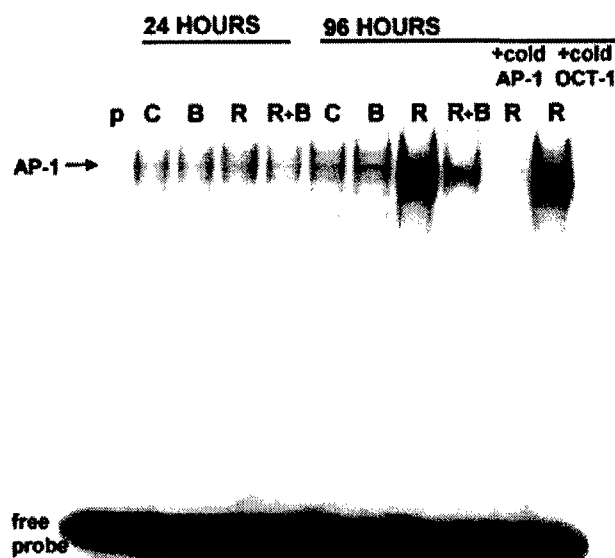

Fig. 3. Effect of R1881 exposure on AP-1 DNA-binding activity in LNCaP cells at 24 and 96 hr. Nuclear proteins were harvested from LNCaP cells exposed to vehicle control medium (C), 5 μM antiandrogen bicalutamide (B), I nM R1881 (R), or I nM R1881 +5 μM bicalutamide (R + B) at 24 and 96 hr and analyzed by electrophoretic mobility shift assay (EMSA) for binding to a ^{32}P-labeled AP-1 consensus oligodeoxynucleotide. The androgen-mediated increase in AP-1 DNA-binding over 96 hr was attenuated by the addition of antiandrogen bicalutamide. Specificity of the AP-1 DNA-binding activity was confirmed by the following controls: addition of excess, non-radiolabeled AP-1 oligodeoxynucleotide to the I nM R1881 sample (+cold AP-1), which resulted in the complete loss of detectable AP-1 DNA binding; addition of non-radiolabled OCT-1 oligodeoxynucleotide with ^{32}P-labeled AP-1 oligodeoxynucleotide to the I nM R1881 sample (+cold OCT-1), which had no effect on the ability to detect the AP-1–DNA complex; and ^{32}P-labeled AP-1 oligodeoxynucleotide incubated with water, i.e., a probe only (p) reaction mix, which did not produce any altered mobility of the oligodeoxynucleotide. Studies were performed twice.
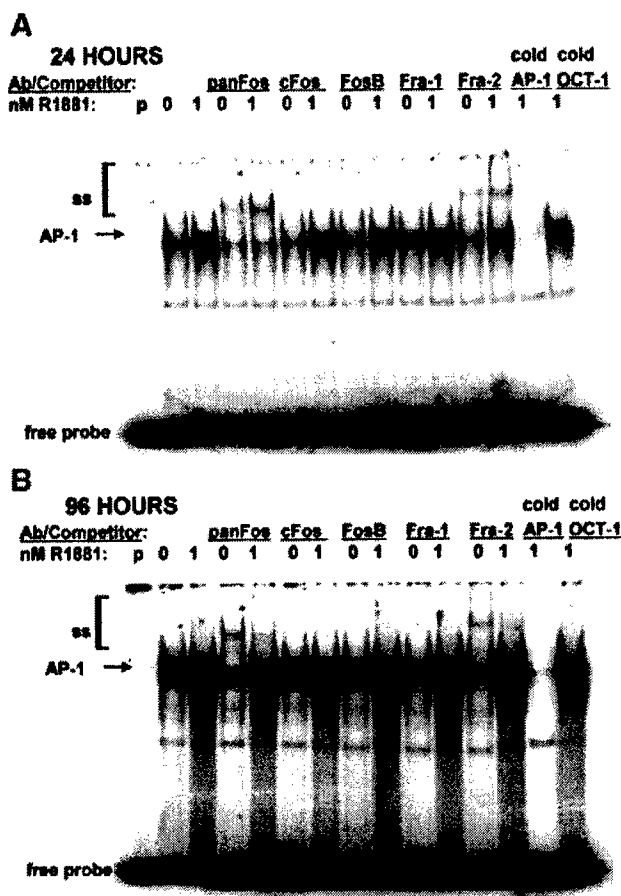
A

24 HOURS   cold cold
Ab/Competitor: panFos cFos FosB Fra-1 Fra-2 AP-1 OCT-1
nM R1881:   p 0 1 0 1 0 1 0 1 0 1 0 1 1 1

B

96 HOURS   cold cold
Ab/Competitor: panFos cFos FosB Fra-1 Fra-2 AP-1 OCT-1
nM R1881:   p 0 1 0 1 0 1 0 1 0 1 0 1 1 1

Fig. 4. Effect of R1881 exposure on AP-1 DNA-binding activity and Fos composition of the AP-1 transcription factor complex in LNCaP cells at 24 and 96 hr. **A**: Nuclear proteins were harvested from LNCaP cells exposed to vehicle control (0) or 1 nM R1881 (1) at 24 hr and analyzed by EMSA for binding to a ^{32}P-labeled AP-1 consensus oligodeoxynucleotide. Analysis of Fos family members in the AP-1 DNA-binding complex were examined by antibody-mediated supershifts (ss). AP-1 DNA-binding was increased by exposure to 1 nM R1881 (1) compared to control cells (0). Antibody to panFos and Fra-2 produced a supershift in both control (0) and 1 nM R1881 (1) exposed cells after 24 hr, with an increased supershift observed after exposure to 1 nM R1881 (1). No supershift was observed following incubation with antibodies to cFos, FosB, or Fra-1. **B**: Analysis of Fos family members (as performed in A) after exposure to 0 (0) or 1 nM R1881 (1) for 96 hr. Antibody to panFos and Fra-2 produced a supershift (ss) in control (0) samples, which was significantly reduced by exposure to 1 nM R1881 (1) for 96 hr. As observed after 24 hr, no supershift was observed following incubation with antibodies to cFos, FosB, or Fra-1. Specificity of the AP-1 DNA-binding activity was confirmed by the following controls in each EMSA: addition of excess, non-radiolabeled AP-1 oligodeoxynucleotide to the 1 nM R1881 sample (cold AP-1), which resulted in the complete loss of detectable AP-1 DNA binding; addition of non-radiolabeled OCT-1 oligodeoxy-nucleotide with ^{32}P-labeled AP-1 oligodeoxynucleotide to the 1 nM R1881 sample (cold OCT-1), which had no effect on the ability to detect the AP-1–DNA complex; and ^{32}P-labeled AP-1 oligodeoxynucleotide incubated with water, i.e., a probe only (p) reaction mix, which did not produce any altered mobility of the oligodeoxynucleotide. Studies were performed three times.

receptor [21]. Androgen exposure did not alter AP-1 DNA-binding activity in DU145 cells [20]. Furthermore, an AP-1 composition analysis of nuclear protein extracts from DU145 cells detected similar binding of JunD, Fra-2, Fra-1, and cJun at both 24 and 96 hr in 1 nM R1881-treated compared to vehicle-treated DU145 cells (data not shown). These results in DU145 cells, along with the attenuation of the androgen-induced AP-1 DNA-binding activity by the antiandrogen bicalutamide, indicate that the AP-1 response in LNCaP cells mediated by androgen exposure is relevant to an androgen receptor signaling pathway and that Fra-2 and JunD contribute significantly to androgen-induced modifications of AP-1 binding.

Androgen-Mediated Changes in junD and fra-2 mRNA Levels in LNCaP Cells

Levels of junD and fra-2 mRNA in LNCaP cells were measured by qRT-PCR. There was no difference in junD mRNA levels between LNCaP cells treated for 24 hr with either vehicle or 1 nM R1881 (Fig. 6A, upper panel). At 96 hr, however, a 3.2-fold increase in junD mRNA was observed in cells exposed to 1 nM R1881 compared to vehicle ($P = 0.05$) (Fig. 6A, upper panel). Levels of fra-2 mRNA were increased 2.0-fold at 24 hr ($P = 0.04$) and 2.6-fold at 96 hr ($P = 0.05$) in 1 nM R1881-treated cells compared to vehicle-treated control cells (Fig. 6A, lower panel).

Androgen-Mediated Changes in JunD and Fra-2 Protein Levels in LNCaP Cells

Levels of JunD and Fra-2 proteins in nuclear extracts of LNCaP cells exposed to vehicle or 1 nM R1881 were compared by immunoblot analysis. There was no difference in JunD nuclear protein levels between vehicle-treated control cells and cells exposed to 1 nM R1881 for 24 hr (Fig. 6B, upper panel). However, at 96 hr a form of JunD protein migrating at approximately 35 kDa was increased in cells exposed to androgen (Fig. 6B, upper panel). Fra-2 protein levels were increased in cells exposed to 1 nM R1881 at both 24 and 96 hr compared to vehicle-treated control cells (Fig. 6B, lower panel).

DISCUSSION

Androgens play a pivotal role in the regulation of prostate cell growth [1–4]. The androgen receptor is the primary mediator of androgenic responses in both normal and malignant prostate cells [5,6]. In this study, we demonstrate that androgen signaling mediated through the androgen receptor can induce AP-1 activity in a human prostate cancer cell model. Specific changes in AP-1 factor binding were found to occur

over time in association with androgen-androgen receptor induction of cellular growth arrest. This study suggests a link between growth changes induced by activated androgen receptor in human prostate cancer cells and specific changes in AP-1 activity.

Consistent with the findings of Horoszewicz et al. [18], we previously demonstrated a dose-dependent, biphasic growth of LNCaP cells exposed to a physiologic range of androgens [13]. After 2–3 days of androgen exposure, concentrations of androgen near 0.05 nM were found to stimulate LNCaP cell growth, whereas concentrations of androgen near 1 nM inhibited LNCaP growth. In the current study, the cellular growth inhibition induced by 1 nM androgen over 96 hr was found to be associated to an arrest of cells in the G_1 phase of the cell cycle without effect on cell viability. Attenuation of the androgen-induced cell growth inhibition and G_1 arrest by the antiandrogen bicalutamide demonstrated that these effects are dependent on activation of the androgen receptor. Our results are consistent with studies reported by Kokontis et al. [22] where sublines of LNCaP cells expressing high androgen receptor levels produced a G_1 cell cycle arrest that

was maximal at 72 hr after androgen exposure. Yuan et al. [27] have also reported that androgen-insensitive PC-3 cells transfected with a functional androgen receptor were growth inhibited on androgen exposure for 72 hr. Thus, androgen induction of signaling for cell growth inhibition is dependent on the level of androgen receptor expressed and the dose of androgen administered. The pathways affected by androgen receptor activation that alter cell growth in prostate cancer cells have not been extensively investigated.

AP-1 factors are key mediators of changes in cell growth [11,28]. We previously demonstrated androgen-mediated changes in AP-1 DNA-binding in LNCaP cells that are both dose- and time-dependent, as are the androgen effects on growth [20]. LNCaP cells exposed to growth-stimulatory concentrations (e.g., 0.05 nM) of androgens were not measurably altered in AP-1 DNA-binding compared to control cells grown in minimal levels of androgen. In contrast, growth-inhibitory concentrations of androgens (e.g., 1 nM) induced a time-dependent increase in AP-1 DNA-binding activity. The maximal increase in AP-1 DNA-binding was observed at 96 hr after androgen exposure [20]. The attenuation of the increase in AP-1 DNA-binding by bicalutamide in the current study demonstrates that activation of the androgen receptor is
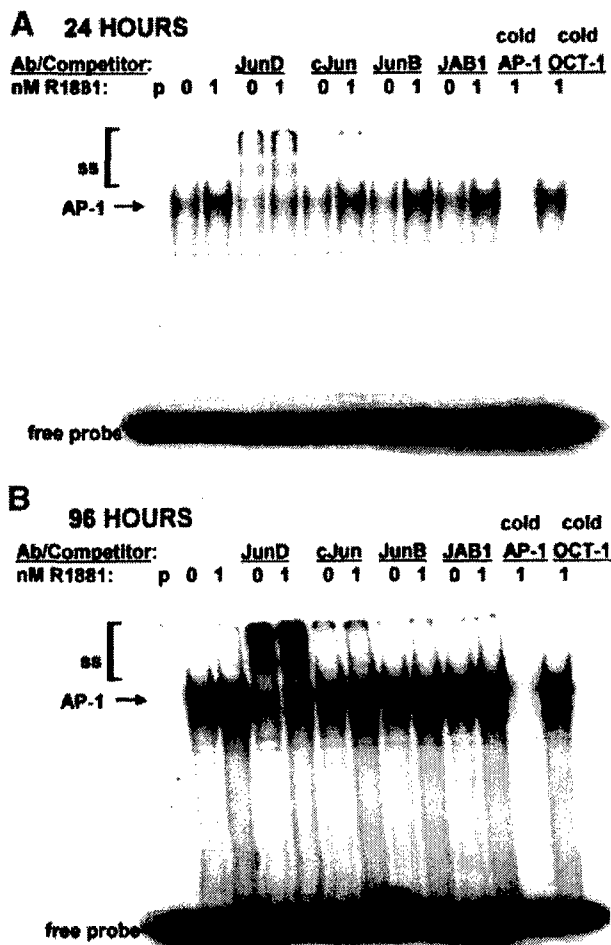

A 24 HOURS

B 96 HOURS

Fig. 5. Effect of RI88I exposure on AP-I DNA-binding activity and Jun composition of the AP-I transcription factor complex in LNCaP cells at 24 and 96 hr. **A**: Nuclear proteins were harvested from LNCaP cells exposed to vehicle control (0) or I nM RI88I (I) at 24 hr and analyzed by EMSA for binding to a ^{32}P-labeled AP-I consensus oligodeoxynucleotide. Analysis of Jun family members in the AP-I DNA-binding complex were examined by antibody-mediated supershifts (ss). AP-I DNA-binding was increased by exposure to I nM RI88I (I) compared to control cells (0). JunD protein was detected in the AP-I-DNA-binding complex for both 0 and I nM RI88I-treated cells, with an increase in JunD binding after exposure to I nM RI88I (I). No supershift was observed after incubation with antibodies for c-Jun, JunB, or JABI. **B**: Analysis of Jun family members (as performed in A) after exposure to 0 (0) or I nM RI88I (I) for 96 hr. AP-I DNA-binding was increased by exposure to I nM RI88I (I) compared to control cells (0) over 96 hr. JunD protein was detected in the AP-I DNA-binding complex for both 0 and I nM RI88I-treated cells and was increased with I nM RI88I exposure (I) for 96 hr. As seen at 24 hr, no supershift was observed after incubation with antibodies for c-Jun, JunB, or JABI. Specificity of the AP-I DNA-binding activity was confirmed by the following controls in each EMSA: addition of excess, non-radiolabeled AP-I oligodeoxynucleotide to the I nM RI88I sample (cold AP-I), which resulted in the complete loss of detectable AP-I DNA binding; addition of non-radiolabeled OCT-I oligodeoxy-nucleotide with ^{32}P-labeled AP-I oligodeoxynucleotide to the I nM RI88I sample (cold OCT-I), which had no effect on the ability to detect the AP-I-DNA complex; and ^{32}P-labeled AP-I oligodeoxynucleotide incubated with water, i.e., a probe only (p) reaction mix, which did not produce any altered mobility of the oligodeoxynucleotide. Studies were performed three times.
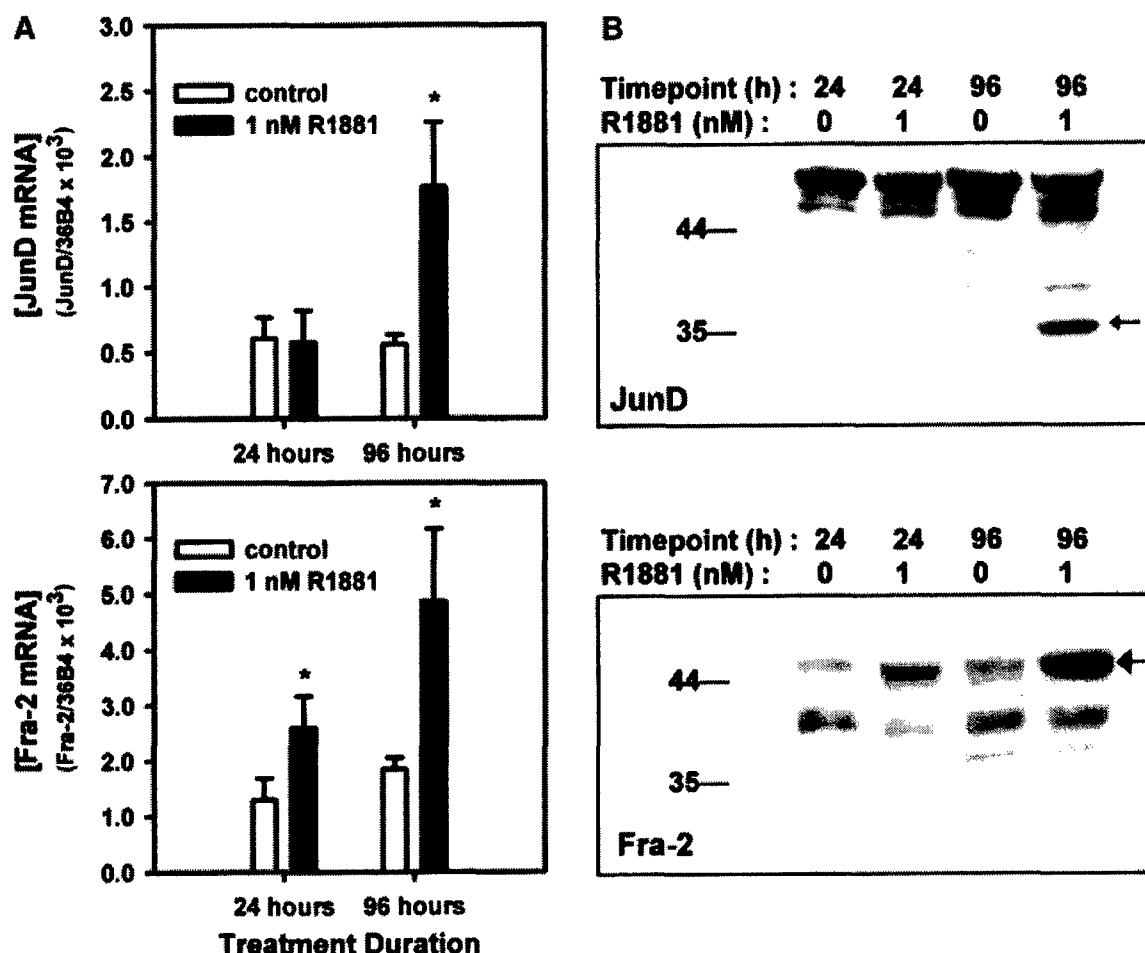
Fig. 6. Effect of R188I exposure on levels of junD and fra-2 mRNA (**A**) and JunD and Fra-2 protein (**B**) in LNCaP cells at 24 and 96 hr. **A:** RNA samples harvested from LNCaP cells exposed to I nM R188I (solid bars) or vehicle control (open bars) at 24 and 96 hr and analyzed by SYBR green I-based quantitative real-time RT-PCR (qRT-PCR) for measurement of junD mRNA levels (**upper panel**) and fra-2 mRNA levels (**lower panel**). Exposure to I nM R188I produced a 3.2-fold increase in junD mRNA by 96 hr. No change in junD mRNA levels was observed after 24 hr of I nM R188I exposure. Compared to the respective vehicle control at each timepoint, exposure to I nM R188I caused an increase in fra-2 mRNA of 2.0-fold by 24 hr and 2.6-fold by 96 hr. For each sample, the mean cDNA copy number determined from triplicate measurements for a particular mRNA species was normalized to the mean cDNA copy number from quadruplicate measurements of ribosomal phosphoprotein 36B4. This mean target to 36B4 mRNA molar ratio for each sample was used in determining the average copy number and associated error in each treatment group. Each data point is the average ± standard deviation of three individual RNA samples. Studies were performed twice. ($*P \leq 0.05$, compared to control at respective timepoint). **B:** Nuclear protein extracts harvested from LNCaP cells exposed to I nM R188I (I) or vehicle control (0) at 24 and 96 hr were analyzed by immunoblot assays using a JunD antibody (**upper panel**) and a Fra-2 antibody (**lower panel**). At 96 hr, a form of JunD migrating near the 35 kDa molecular weight standard was increased due to exposure to I nM R188I (small arrow). At both 24 and 96 hr, Fra-2 protein migrating near the 44 kDa molecular weight standard was increased by I nM R188I exposure (large arrow). Studies were performed three times. Protein sizes were estimated by the bands of the molecular weight standard marker, which are shown schematically on the left.

necessary for androgen induction of AP-1 DNA-binding. In this study, changes in the AP-1 complex in LNCaP cells exposed to 1 nM androgen were examined at two key time points: at 24 hr, which allowed the evaluation of earlier events following androgen receptor activation by androgen exposure, and at 96 hr, when the androgen treated cultures showed maximal effects on AP-1 binding in previous studies.

AP-1 factors mediate transcriptional changes in normal cell growth and in carcinogenesis [7]. In the current study, a luciferase enzyme reporter vector driven by AP-1 response elements was used to measure changes in AP-1 driven transcriptional activity. No effects on AP-1 activity were apparent after androgen exposure for 24 hr. However, after 96 hr, AP-1 transcriptional activity was increased nearly 5-fold.

The antiandrogen bicalutamide significantly inhibited AP-1 activation by androgen at 96 hr, suggesting an integral role of the androgen receptor in androgen-induced AP-1 activation. Therefore, changes in AP-1 transcriptional activity after androgen exposure in LNCaP cells were found to be time- and androgen receptor-dependent.

The cellular response to AP-1 signaling depends on the transcriptional activity and dimer composition of the AP-1 DNA-binding complex, consisting of Fos and Jun family members [7,9,10]. Specific AP-1 family members have been shown to have an integral role in androgen-regulated prostate homeostasis. For example, Feng et al. [29] found that Fos was necessary for castration-induced apoptosis of the prostate gland. Previously, we found that c-Fos protein levels were decreased while c-Jun protein levels were unaffected at both 24 and 96 hr in nuclear extracts from 1 nM R1881-exposed LNCaP cells [20]. In the current study, EMSA and antibody supershift assays were performed to assess changes in Fos/Jun composition of the AP-1 complex in LNCaP cells exposed to androgen for 24 and 96 hr. Analysis using antibodies for individual Fos family members led to the identification of Fra-2 as a protein whose involvement in the AP-1 DNA-binding complex was time-dependent after androgen exposure in LNCaP cells, with a decrease in Fra-2 protein binding observed over the 24–96 hr time period after androgen exposure. Analyzes with antibodies for individual Jun family members detected JunD in the AP-1 DNA-binding complex in both control- and androgen-treated cells at 24 and 96 hr after initiation of androgen treatment. Significantly more JunD binding was observed at 96 hr than at 24 hr after androgen exposure. Therefore, we have determined that androgen exposure can modulate AP-1 activity and AP-1 DNA-binding in LNCaP human prostate carcinoma cells with a loss of Fra-2 involvement and emergence of JunD as a dominant component of the AP-1–DNA complex over 96 hr.

Based on the observed changes in the composition of the AP-1 DNA-binding complex from 24 to 96 hr, we hypothesized that androgen exposure would lead to an increase in cellular levels of JunD protein and/or a decrease in Fra-2 protein over this time period. Immunoblot analysis showed an increase in JunD protein at 96 hr and an increase in Fra-2 protein at 24 and 96 hr in nuclear extracts from androgen-treated LNCaP cells. The increase in Fra-2 protein correlated with an increase in fra-2 mRNA levels, indicating that the increased Fra-2 protein level could result, at least in part, from increases in mRNA abundance encoding the Fra-2 protein. Interestingly, Fra-2 protein levels continued to increase over 96 hr after androgen exposure even though EMSA analysis did not demonstrate a significant contribution of Fra-2 to the AP-1 complex at

96 hr. Androgen exposure also led to an increase in JunD protein over 96 hr that correlated with increased junD mRNA abundance. Thus, demonstrable changes in Fra-2 and JunD AP-1 family members were induced by androgen exposure in LNCaP cells. However, binding of individual AP-1 family members to the AP-1 complex was not necessarily proportional to their nuclear levels.

We observed a 35 kDa form of JunD and a 44 kDa form of Fra-2 to be increased by androgen exposure. Regulation of AP-1 proteins can occur via post-transcriptional modifications [30]. Two isoforms of JunD protein have been reported, which are encoded from the same mRNA transcript and are produced by an additional internal translation AUG-initiation site [31]. The predicted molecular weights of these two JunD isoforms are 35 and 40 kDa [31]. The observed molecular weights for JunD are reported in the range of 34–44 kDa, including multiple forms that result from JunD phosphorylation, proteolytic degradation, and/or additional isoforms generated from alternative translation initiation sites [31–35]. Our results suggest that androgen exposure can mediate an increase in the fast migrating form of JunD. Fra-2 protein is reported to have a calculated molecular weight of 35 kDa and migrates in the range of 35–55 kDa [32,36–40]. We observed two major Fra-2 protein bands in this range and the slower migrating species, near the 44 kDa marker, was consistently increased in androgen-treated cells. Multiple bands have been observed for Fra-2 in other cell types, largely due to phosphorylation differences but potentially also representing two isoforms resulting from alternative splicing as suggested by Andreucci et al. [40]. Therefore, androgen exposure in LNCaP cells led to increased junD and fra-2 mRNA levels and specific isoforms of the Fra-2 and JunD proteins.

Although it is uncertain how the changes in JunD and Fra-2 may contribute to the androgen-induced growth changes in LNCaP cells in the current study, other groups have reported that JunD and Fra-2 are relevant in maintaining functional, differentiated cells. For example, JunD and Fra-2 have been associated with growth inhibition/differentiation induced by various stimuli in a wide variety of cell types, such as mouse fibroblasts [32,41]; rat pheochromocytoma [38], muscle cells [40], and osteoblasts [42]; and human chronic myelogenous leukemia cells [43], intestinal epithelial cells [44], and ovarian cancer cells [45]. We have previously reported other characteristics of LNCaP cells induced by 1 nM androgen exposure that are suggestive of a more differentiated phenotype, including elevated levels of cytokeratin 18 protein, which is expressed in more differentiated luminal prostate cells, and increased secretion of prostate-specific antigen

protein [46]. In the current study, we found that an androgen-mediated increase in JunD and loss of Fra-2 association in the AP-1 DNA-binding complex is associated with G_1 arrest cell growth inhibition that is consistent with a differentiated phenotype. Thus, we hypothesize that JunD and Fra-2 may be important factors in an androgen–androgen receptor signaling pathway that supports a differentiated phenotype in androgen-sensitive prostate cells.

In summary, we report that androgen activation of the androgen receptor can induce AP-1 activity with specific changes in the composition of the AP-1 DNA-binding complex over time in the androgen-responsive LNCaP human prostate carcinoma cell model. Increased AP-1 DNA-binding over 24 hr in cells exposed to androgen is associated with increased Fra-2. Further increased AP-1 DNA-binding by 96 hr is associated with increased JunD, specifically in expression of a 35 kDa isoform. To the best of our knowledge, this is the first report of the enhancement of expression of the 35 kDa isoform of JunD by androgen exposure. Surprisingly, Fra-2 binding in the AP-1 complex decreases over 96 hr, in paradox to a further increase in nuclear levels of Fra-2 in androgen-exposed cells. As the decrease in Fra-2 and the increase in JunD in the AP-1 complex occur over a relatively long period of time (i.e., 96 hr) after androgen exposure, it is unlikely that either androgen alone or its complex with androgen receptor is acting as a cofactor in JunD association with the AP-1 site. Thus, the replacement of Fra-2–AP-1 complex with JunD–AP-1 complex is either due to an induction of a yet unknown cofactor by androgen or due to a relatively higher affinity of the 35 kDa isoform of JunD for the AP-1 binding site as compared to that of Fra-2. Proteomics studies are being designed to identify specific alterations in proteins bound to the AP-1 DNA-binding site over time. Importantly, these changes are associated with G_1 cell cycle arrest and cell growth inhibition consistent with differentiation. We speculate that the replacement of the Fra-2–AP-1 complex with a JunD–AP-1 complex is an important factor in the induction of a differentiated phenotype of the prostate cancer cells that could be an exciting target for prevention as well as treatment of prostate cancer.

ACKNOWLEDGMENTS

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Appendix 4

Thompson TA and Wilding G. Androgen antagonist activity by the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol in human prostate carcinoma cells. Molecular Cancer Therapeutics 2:797-803, 2003.

Androgen Antagonist Activity by the Antioxidant Moiety of Vitamin E, 2,2,5,7,8-Pentamethyl-6-chromanol in Human Prostate Carcinoma Cells[1]

Todd A. Thompson and George Wilding[2]

University of Wisconsin Comprehensive Cancer Center [T. A. T., G. W.] and University of Wisconsin Department of Medicine [G. W.], University of Wisconsin-Madison, Madison, Wisconsin 53792

Abstract

Antioxidants, such as vitamin E, are being investigated for efficacy in prostate cancer prevention. In this study, we show that the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol (PMCol), has antiandrogen activity in prostate carcinoma cells. In the presence of PMCol, the androgen-stimulated biphasic growth curve of LNCaP human prostate carcinoma cells was shifted to the right. The PMCol-induced growth shift was similar to that produced by treatment with the pure antiandrogen bicalutamide (i.e., Casodex), indicative of androgen receptor (AR) antagonist activity. The concentration of PMCol used was below the concentration required to affect cell growth or viability in the absence of androgen. Using an AR binding competition assay, PMCol was found to be a potent antiandrogen in both LNCaP and LAPC4 cells, with an IC_{50} of approximately 10 μM against 1 nM R1881 (methyltrienolone; a stable, synthetic androgen). Prostate-specific antigen release from LNCaP cells produced by androgen exposure with either 0.05 or 1.0 nM R1881 was inhibited 100% and 80%, respectively, by 30 μM PMCol. Also, PMCol inhibited androgen-induced promoter activation in both LNCaP and LAPC4 cells. However, PMCol did not affect AR protein levels, suggesting that the inhibitory effects of PMCol on androgenic pathways were not due to decreased expression of the AR. Therefore, growth modulation by the antioxidant moiety of vitamin E in androgen-sensitive prostate carcinoma cells is due, at least in part, to its potent antiandrogenic activity.

Introduction

The activity of androgens is tissue specific and mediated through the AR.[3] The disruption of androgens and AR activity alters the regulation of androgen-sensitive tissues, such as the prostate gland (1). In the prostate, androgens have a central role in normal glandular development and function (2). However, androgens are also necessary for the development of prostate cancer. The role of androgens in prostate cancer development is emphasized by the observation that eunuchs and men that have a mutation in 5α-reductase type II, an enzyme that converts testosterone to the more potent dihydrotestosterone, do not develop prostate cancer (3). The incidence of prostate cancer has continued to rise for the last two decades, currently affecting over 200,000 men in the United States each year (4). Agents that permit the necessary actions of androgen for normal tissue function while reducing the role of androgens in the pathogenesis of androgen-sensitive tissues may serve as a useful means of reducing prostate cancer development. Recently, several agents have been reported to prevent prostate cancer development, such as selenium, lycopene, and vitamin E (5). Due to the biochemical nature of these agents, they are believed to act primarily through antioxidant-related pathways. However, the scope of their biological activity has not been extensively investigated.

Vitamin E is a family of naturally occurring dietary factors, which were originally identified as necessary for reproduction (6). α-Tocopherol, the most potent form of vitamin E, has two main components, a 16-carbon phytyl chain and a chromanol moiety with four methyl group substitutions (7). Biologically, α-tocopherol is thought to act primarily as an antioxidant, reducing oxidative damage to lipids. The chromanol moiety of α-tocopherol is responsible for its antioxidant activity, whereas the phytyl chain increases the lipophilicity of α-tocopherol and contributes to its tissue and subcellular distribution (8). Cell culture studies using α-tocopherol are difficult to perform due to its limited water solubility. However, the antioxidant chromanol moiety of α-tocopherol, PMCol, which does not possess a phytyl chain, is sufficiently water soluble to permit studies in cell culture.

Most human prostate carcinoma cell lines are androgen independent. The LNCaP human prostate carcinoma cell line is one of the few cell lines to show demonstrable responses to androgen exposure (9). Interestingly, LNCaP cells produce a biphasic growth response to androgen exposure, with growth stimulation occurring at lower doses and growth

[3] The abbreviations used are: AR, androgen receptor; CSS, charcoal-stripped serum; MMTV, mouse mammary tumor virus; PMC, 2,2,5,7,8-pentamethylchroman; PMCol, 2,2,5,7,8-pentamethyl-6-chromanol; PSA, prostate-specific antigen.
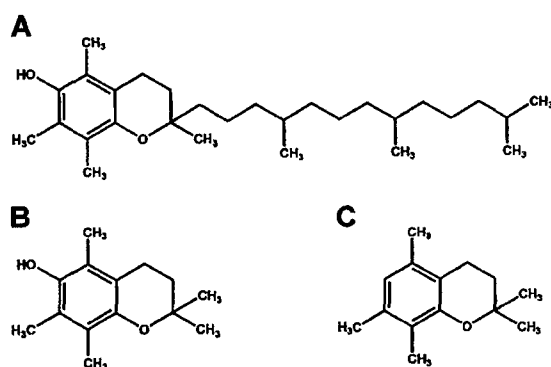
Fig. 1. Structure of vitamin E (i.e., α-tocopherol) and related compounds. A, α-tocopherol. B, PMCol. C, PMC.

inhibition occurring in the absence of androgen or in the presence of high androgen levels (9, 10). In addition, a number of androgen-sensitive responses are induced in LNCaP cells. For example, LNCaP cells produce a dose-dependent increase in PSA expression on androgen exposure (11, 12). Also, androgen-sensitive promoters, such as the MMTV promoter, are activated by androgen in LNCaP cells (13). The exquisite sensitivity of LNCaP cells to androgenic stimulation may be due to a mutation in the ligand-binding domain of the AR (14). To date, the LNCaP prostate cell line has been the most extensively characterized prostate cell line for examining the effects of androgens. More recently, the LAPC4 cell line has been introduced as another androgen-sensitive human prostate carcinoma cell line that expresses a normal AR (15). However, the response of LAPC4 cells to androgens is not as pronounced as that observed in LNCaP cells. Collectively, the LNCaP and LAPC4 human prostate carcinoma cell lines provide valuable models for investigating androgen-regulated cellular pathways.

Studies on the actions of vitamin E and vitamin E analogues on prostate carcinoma cells have only recently begun. Previous studies have focused primarily on the inhibition of prostate cell growth by vitamin E treatment, which may occur through effects on cell cycle regulators (16–18). Apoptotic responses induced by vitamin E treatment have also been observed in LNCaP cells (19, 20). Interestingly, vitamin E-induced apoptotic responses were enhanced by coadministration of androgen (19). Zhang et al. (21) reported that vitamin E succinate reduces the levels of the AR in LNCaP cells, with resultant inhibition of androgen-mediated responses. However, the direct actions of vitamin E and related compounds on AR activity in prostate cells have not been extensively examined. In the current study, the AR antagonist activity and modulation of androgen-sensitive pathways by the vitamin E derivative PMCol were investigated in human prostate carcinoma cells.

Materials and Methods

Chemicals. PMCol and PMC were obtained from Aldrich (Milwaukee, WI). The chemical structures of α-tocopherol, PMCol, and PMC are shown in Fig. 1. Bicalutamide (Casodex) was kindly provided by AstraZeneca Pharmaceuticals

(Wilmington, DE). R1881 (methyltrienolone) and ^{3}H-R1881 (87 Ci/mmol) were obtained from Perkin-Elmer/New England Nuclear Life Science Products (Boston, MA). All other chemicals used in these studies were acquired from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. LNCaP cells were acquired from American Type Culture Collection (Manassas, VA), and LAPC4 cells were kindly provided by Dr. Robert Reiter (University of California-Los Angeles) and maintained in DMEM containing 5% heat-inactivated FCS (Sigma) with streptomycin-penicillin antibiotics (designated DMEM/fetal bovine serum) in a 5% CO_2 incubator at 37°C. For experiments evaluating androgenic responses, cells were cultured in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 4% charcoal-stripped FCS and 1% unstripped FCS (designated DMEM/CSS).

AR Binding Competition Assay. An AR binding competition assay was performed as described previously (22). LNCaP or LAPC4 prostate carcinoma cells were plated in 12-well tissue culture dishes (Costar, Corning, NY) at 3.0 × 10^5 cells/well in phenol-red free DMEM/CSS 3 days before analysis. For competitor analysis, DMEM/CSS was removed by aspiration and replaced with 1 ml of phenol-red free DMEM containing 1 nM ^{3}H-R1881, 1 μM triamcinolone acetonide, and competitor at the specified concentrations for 2 h at 37°C in a 5% CO_2 incubator. After incubation, competitor solution was aspirated, and cells were removed from the plate by trypsinization and placed in 12 × 75-mm polystyrene tubes. Cells were washed twice with 1 ml of phenol red-free DMEM and placed in 8.0 ml of ScintiVerse II Scintillation Cocktail (Fisher Scientific, Pittsburgh, PA) for determination of radioactivity (i.e., dpm) using a Beckman LS 6000TA Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA).

Cell Growth and Viability Analyses. Five thousand LNCaP or LAPC4 cells were plated in each well of 96-well plates (Costar) in 100 μl of DMEM/CSS. Two to 3 days after plating, cells were treated by adding 100 μl of DMEM/CSS containing 2× the concentration of the specified treatment to each well. Four days after treatment, the relative cell number was estimated by determining the DNA concentration of each well using a Hoechst-based fluorescence DNA assay, as described previously (23). Growth analysis with DU145 cells was performed in a manner similar to that described for LNCaP and LAPC4 cells, except that DU145 cells were initially seeded at 500 cells/well. Cell viability was determined by trypan blue exclusion and quantified by light microscopic analysis using a hemacytometer.

Determination of Secreted PSA Levels. LNCaP cells were cultured in 96-well plates (Costar) at 5000 cells/well in DMEM/CSS 1 day before treatment. Forty-eight h after treatment, PSA levels in cell culture media were determined using the Tandem-MP PSA kit (Beckman Coulter, Inc.) according to the manufacturer's instructions. PSA levels were normalized to DNA levels as determined using a Hoechst-based fluorescence DNA assay (23).

Androgen-stimulated Promoter Reporter Assay Analysis. LNCaP and LAPC4 prostate carcinoma cell lines were cultured in 12-well cell culture plates (Costar) in DMEM/CSS

2–3 days before transfection. Androgen-induced trancriptional activation was determined using a reporter construct with a MMTV promoter that regulates the expression of luciferase (24). LNCaP and LAPC4 cells were transfected with the MMTV/luciferase plasmid using the Effectene Transfection Reagent (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Twenty-four h after transfection, cells were treated with R1881 with or without test reagents at the specified concentrations. Cell extracts were acquired 24–48 h after treatment by removing medium, washing 1× with PBS, and obtaining extract with 200 μl of 1× Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was determined as described previously (24).

Immunoblot Analysis of AR Protein Levels. LNCaP cells were plated at a density of 1 × 10^6 cells/100-mm cell culture plate in 10 ml of DMEM/fetal bovine serum and maintained in incubators at 37°C in 5% CO_2. After 5 days of treatment with vehicle, 30 μM PMC, 30 μM PMCol, or 1.0 μM bicalutamide, cells were washed in cold 1× PBS and lysed in a buffer containing 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin in 1× PBS. Total protein (10 μg) from cell extracts was electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a GENIE wet transfer system (Idea Scientific, Minneapolis, MN). Membranes were blocked in Tris-buffered saline containing 5% nonfat dry milk and then incubated with mouse anti-AR monoclonal antibody (441; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse antiactin antibody (A5441; Sigma). Membranes were then incubated with a secondary horseradish peroxidase-conjugated antimouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed using Enhanced Chemiluminescence Plus reagent (Amersham Pharmacia Biotech). Autoradiograms were prepared by exposing the blots to BioMax Light X-ray film (Eastman Kodak Co., Rochester, NY) and developed using a CURIX 60 CP Processor (Agfa, Ridgefield Park, NJ).

Statistical Analysis. Significant differences in values between groups were assessed using a two-sided Student's *t* test. *P*s less than 0.05 were used to signify statistical significance.

Results

PMCol Inhibits Androgen Binding in Prostate Cancer Cells. AR competition was determined using ^{3}H-R1881 in the androgen-sensitive LNCaP cell line, which expresses a functional mutant AR (25), and the LAPC4 cell line, which express a normal human AR (15). Increasing concentrations of the AR antagonist bicalutamide were found to progressively inhibit R1881 binding (Fig. 2A), with an estimated IC_{50} of 0.7 μM in LNCaP cells. PMCol was found to be approximately 10-fold less potent at competing for ^{3}H-R1881 than bicalutamide in LNCaP cells, with an estimated IC_{50} of 7.2 μM (Fig. 2A). Repeated studies of PMCol competition for ^{3}H-R1881 binding gave IC_{50} values ranging from 5 to 15 μM (data not shown). In contrast, PMC, in which the 6-hydroxyl of PMCol is absent, had less antiandrogenic activity than PMCol (Fig. 2A) and significantly reduced cell viability at a
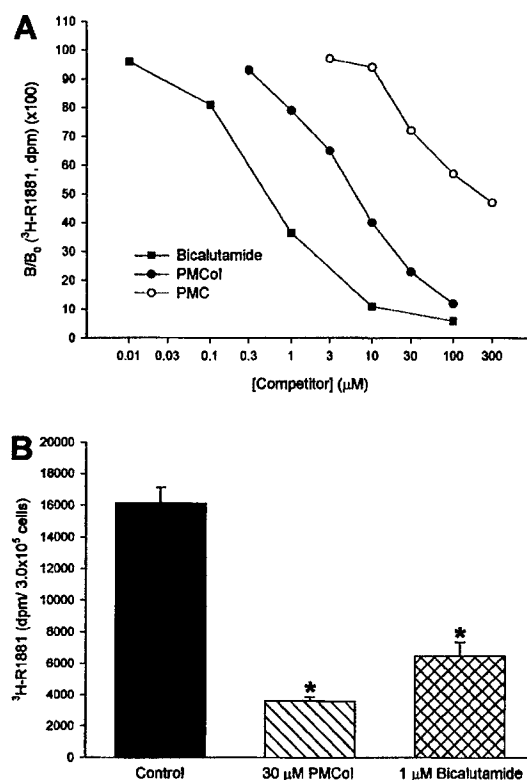

Fig. 2. PMCol competition analysis of R1881 binding in human prostate carcinoma cells. *A*, dose response for the competition of PMCol, PMC, and bicalutamide for AR binding to ^{3}H-R1881 was determined in LNCaP cells. *B*, competition for ^{3}H-R1881 binding in LAPC4 cells was determined for 30 μM PMCol and 1 μM bicalutamide. *, *P* < 0.05; *n* = 4.

concentration of 100 μM within 2 h of treatment (data not shown). Based on the R1881 competition results in LNCaP cells (Fig. 2A), a dose of 30 μM PMC and PMCol was used in most of these studies, allowing an effective comparison of the antiandrogenic activity between PMC and PMCol. In LAPC4 cells, treatment with 30 μM PMCol produced a 75% decrease in ^{3}H-R1881 binding, and treatment with 1 μM bicalutamide produced a 62% decrease in ^{3}H-R1881 binding (Fig. 2B).

Modulation of Prostate Carcinoma Cell Growth and Viability by PMCol. Changes in growth of the androgen-independent DU145 prostate carcinoma cell line and the androgen-sensitive LNCaP and LAPC4 prostate cell lines were assessed at concentrations of PMCol ranging from 10 to 100 μM (Fig. 3A). Concentrations of 50, 60, and 80 μM PMCol were required to significantly reduce cell growth in LNCaP, LAPC4, and DU145 cells, respectively (Fig. 3A). LNCaP cells produce a biphasic growth response to androgen exposure (9). Modulation of LNCaP cell growth by PMCol treatment was examined over 4 days. PMCol had no growth-modulatory activity in vehicle control-treated LNCaP cells grown in androgen-deficient media (*i.e.*, PMCol did not have AR agonist activity) at concentrations ranging from 10 to 30 μM PMCol (Fig. 3B). However, LNCaP cell growth was decreased at concentrations equal to or higher than 40 μM
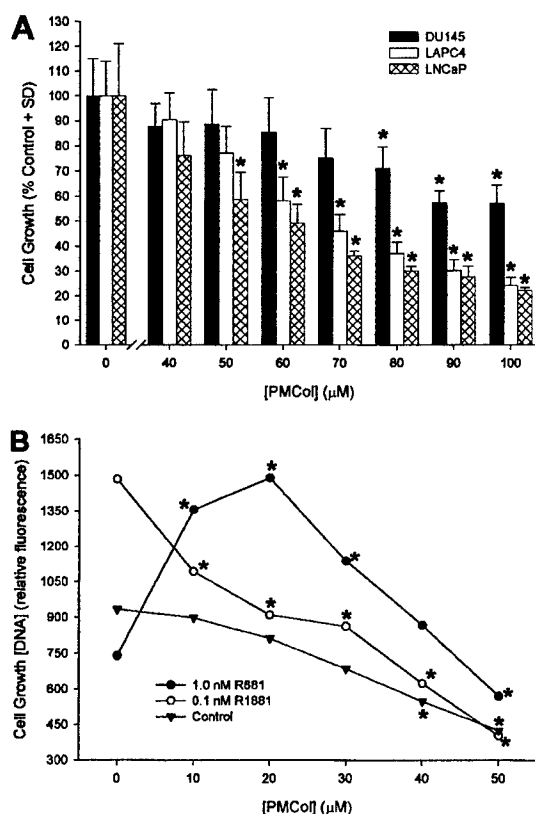
Fig. 3. Growth modulation of human prostate carcinoma cells by PMCol. A, dose response of DU145, LAPC4, and LNCaP cells grown in medium containing 5% serum measured 4 days after PMCol treatment. Treatment with 50 μM PMCol significantly reduced LNCaP prostate cell growth, whereas a concentration of 80 μM PMCol was required to significantly decrease growth in the androgen-independent DU145 prostate cell line (*, P < 0.05). B, the PMCol dose response of LNCaP cell growth was determined in cells exposed to androgen-deficient conditions (i.e., using medium containing reduced androgen levels) with or without the addition of a growth-stimulatory dose of 0.05 nM R1881 or a growth-inhibitory dose of 1.0 nM R1881. *, significantly different than 0 μM PMCol-treated cells, P < 0.05; n = 6.

PMCol (Fig. 3B), and PMCol concentrations of ≥100 μM produced significant cell death at 48 and 96 h (Table 1). Stimulation of LNCaP growth by exposure to 0.1 nM R1881 was significantly inhibited by treatment with concentrations of ≥10 μM PMCol (Fig. 3B). However, a significant stimulation in LNCaP cell growth was observed in the presence of a normally growth-inhibitory concentration of 1.0 nM R1881 with treatment of 10–30 μM PMCol (Fig. 3B). The R1881-stimulated growth curve of LNCaP cells was shifted to the right in the presence of 30 μM PMCol, similar to that produced by treatment with 1 μM bicalutamide (Fig. 4). A more modest, but significant, shift to the right in the androgen-induced LNCaP growth curve was observed by treatment with 30 μM PMC (Fig. 4).

Inhibition of PSA Secretion by PMCol in LNCaP Cells. PSA secretion by LNCaP cells is stimulated by androgen exposure in a dose-dependent manner (12). The R1881-stimulated production of PSA from LNCaP cells was measured after PMCol treatment for 48 h. PSA release from

LNCaP cells was not affected by treatment with 30 μM PMCol alone (Fig. 5). However, PSA levels were increased 3.1-fold after exposure to a growth-stimulatory dose of 0.05 nM R1881, which was completely inhibited by treatment with 30 μM PMCol (Fig. 5). Exposure of LNCaP cells to 1.0 nM R1881 produced a 12-fold increase in PSA levels by 48 h, which was decreased 20%, 81%, and 43% by treatment with 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide, respectively (Fig. 5).

Inhibition of Androgen-stimulated Transcriptional Activation by PMCol. Studies on androgen-regulated transcriptional activation were performed in LNCaP and LAPC4 cells transiently transfected with a reporter vector that uses the androgen-sensitive MMTV long terminal repeat to drive expression of a luciferase reporter gene. In LNCaP cells, PMCol treatment alone had no effect on MMTV promoter activity, whereas luciferase expression was increased 54-fold after exposure to 1.0 nM R1881 for 24 h (Fig. 6A). Luciferase expression induced by exposure to 1.0 nM R1881 in LNCaP cells for 24 h was decreased 50% and 70% by treatment with 25 and 50 μM PMCol, respectively (Fig. 6A). Similarly, LAPC4 cells exposed to 1.0 nM R1881 produced a 20-fold increase in MMTV long terminal repeat-driven luciferase expression that was decreased 60% by treatment with 30 μM PMCol after 24 h (Fig. 6B). In both LNCaP and LAPC4 cells, treatment with 1 μM bicalutamide decreased 1.0 nM R1881-stimulated luciferase expression approximately 50% (Fig. 6, A and B).

AR Protein Levels in PMCol-exposed LNCaP Cells. Previous studies in LNCaP cells have reported that AR levels are decreased after treatment with vitamin E analogues, which may account for the reduced sensitivity of these cells to androgen exposure (21). However, in the current study, treatment with 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide for 5 days did not result in altered AR protein levels in LNCaP cells (Fig. 7).

Discussion

In the current study, we examine the effects of an agent traditionally considered as an antioxidant on prostate carcinoma cells. Epidemiological studies provide intriguing evidence that antioxidant dietary factors such as β-lycopene and vitamin E may help prevent prostate cancer development (5). Although these agents have been classified as antioxidants, the mechanism by which they may contribute to prostate cancer prevention has not been firmly established. Androgens are known to have an essential role in prostate cancer development (3). Modulation of androgen activity may provide a means of prostate cancer prevention (26). Here, we report the antioxidant moiety of vitamin E, PMCol, to be a potent antiandrogen in androgen-sensitive human prostate carcinoma cells.

The LNCaP human prostate carcinoma cell line is one of the few prostate cell lines that show demonstrable physiological changes resulting from androgen exposure, such as growth modulation (9). Therefore, the LNCaP cell line has proven valuable in identifying agents that alter androgen-stimulated cell growth. In the current study, PMCol shifted the androgen-mediated growth curve in LNCaP cells such

Table 1 Time- and dose-dependent changes in LNCaP cell viability after PMCol treatment

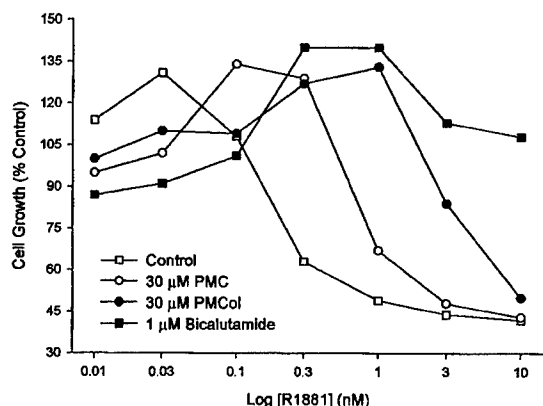| Time (h) | % Cell viability[a] (SD) | | | | | |
| | PMCol (μM) | | | | | |
	0	25	50	75	100	250
48	92.3 (4.7)	90.0 (2.8)	88.0 (3.4)	80.0 (12.5)	71.0 (8.7)[b]	11.0 (8.6)[b]
96	88.0 (2.5)	87.0 (4.8)	85.0 (4.6)	87.0 (4.0)	21.0 (3.3)[b]	2.0 (1.8)[b]

[a] Determined by trypan blue exclusion analysis and quantified using a hemacytometer.
[b] Significantly different compared to 0 μM PMCol (P <0.05; n = 4).

Fig. 4. Shifts in the R1881-stimulated biphasic LNCaP growth response were determined after treatment with 30 μM PMCol, 30 μM PMC, or 1 μM bicalutamide for 4 days. The inhibition of growth response is readily apparent at 0.3 nM R1881 exposure, where LNCaP growth from PMCol, PMC, and bicalutamide treatment was equivalent to the growth response in control cells produced by exposure to only 0.03 nM R1881.
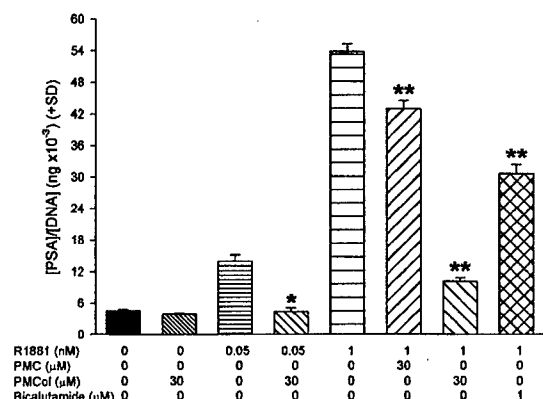

Fig. 5. Analysis of PMCol effects on androgen-induced PSA secretion from LNCaP cells. PSA secretion was determined 48 h after exposure to a growth-stimulatory dose of 0.05 nM R1881 or a growth-inhibitory dose of 1.0 nM R1881 in the presence of 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide. *, P < 0.05 compared with 0.05 nM R1881-treated cells; **, P < 0.05 compared with 1.0 nM R1881-treated cells; n = 3.

Fig. 6. Androgen-induced MMTV promoter activity in LNCaP (A) and LAPC4 (B) cells after PMCol treatment. A, the effects of 25 μM PMCol, 50 μM PMCol, and 1 μM bicalutamide treatment for 24 h on MMTV promoter activity induced by R1881 were assessed in LNCaP cells. B, LAPC4 cells exposed to 30 μM PMCol effectively inhibited androgen-induced MMTV promoter activity. *, P < 0.05; n = 4.

that higher androgen concentrations were necessary to produce the biphasic growth response typically observed in LNCaP cells. The LNCaP growth shift with PMCol treatment was sufficient to produce growth stimulation in the presence of 1.0 nM R1881, a concentration of R1881 that typically inhibits LNCaP proliferation (10). The shift in LNCaP growth
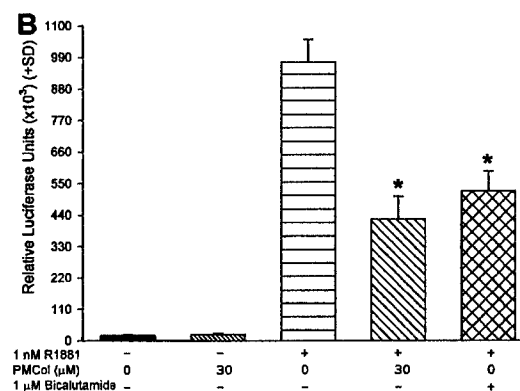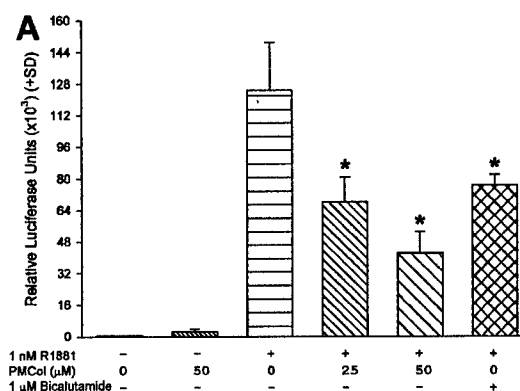
pattern observed with PMCol treatment was similar to that observed in LNCaP cells after treatment with the pure anti-androgen bicalutamide. Also, the IC_{50} of PMCol observed in an androgen competition analysis for R1881 binding in LNCaP cells is in agreement with the dose-response shift in androgen-mediated growth of LNCaP cells after PMCol treatment. Together, these results suggest that the shift observed in the androgen-modulated growth of LNCaP cells was due to the antiandrogenic activity of PMCol.

Although LNCaP cells have proven to be useful in evaluating androgen-responsive pathways, the use of LNCaP cells
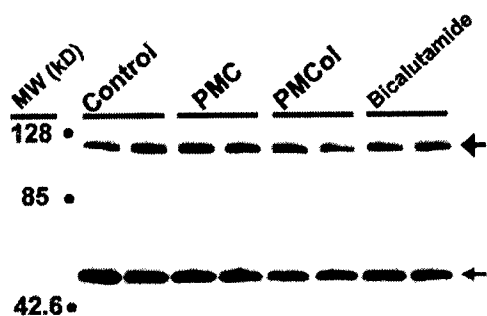
Fig. 7. Immunoblot analysis of AR protein levels. AR protein levels were not significantly altered in LNCaP cells exposed to 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide for 5 days compared with AR levels in vehicle control-exposed cells. LNCaP cells were grown in medium containing 5% serum to provide endogenous serum androgens, thus allowing antiandrogenic modulation of AR protein levels. The *large arrow* points to AR protein bands, and the *small arrow* points to β-actin protein bands.

to assess antiandrogenic activity can be inaccurate because LNCaP cells harbor a mutant AR (25). The AR in LNCaP cells, although functional, has been reported to have altered ligand binding affinity (14) and is stimulated by some agents that are antagonists for the wild-type AR (22). Therefore, in this study, competition for AR binding by PMCol was also assessed in the LAPC4 human prostate carcinoma cell line, which expresses a wild-type AR (15). PMCol competition for R1881 binding was found to be similar for LNCaP and LAPC4 cells. In addition, the pure antiandrogen bicalutamide was found to have equivalent AR competition activity in LNCaP and LAPC4 cells. Therefore, the pure antiandrogen bicalutamide and PMCol were found to possess comparable AR antagonist activity in LNCaP cells, which express a functional mutant AR, and LAPC4 cells, which express a normal AR.

The AR functions primarily as a transcription factor that is activated by androgen binding (1). In these studies, the androgen-responsive MMTV promoter was used to assess modulation of androgen-stimulated transcriptional activity. Upon androgen exposure (*i.e.*, R1881), MMTV promoter activity was stimulated in both LNCaP and LAPC4 cells. Also, in both cell lines, R1881 stimulation of MMTV activity was significantly inhibited by PMCol treatment. PMCol treatment alone did not stimulate MMTV promoter activity (*i.e.*, PMCol was not found to have AR agonist or partial agonist activity). The effects of androgen exposure on transcriptional activation were further observed by the inhibition of androgen-stimulated PSA release after treatment with PMCol in LNCaP cells. Previously, vitamin E succinate was reported to inhibit the effects of androgen on LNCaP cells through down-regulation of AR levels (21). Other agents, such as curcumin, have been shown to decrease AR expression in LNCaP cells (27). In the current study, treatment of LNCaP cells with 30 μM PMCol for 5 days did not affect AR protein levels. Therefore, PMCol was found to be a potent inhibitor of transcriptional activation of androgen-responsive promoters, likely through directly blocking of AR activation by androgen.

Understanding how structure-activity relationships of the chromanol ring of PMCol contribute to antiandrogenic activity may prove useful in developing potent chromanol ring-

based nonsteroidal antiandrogenic agents. In the current study, PMC, which lacks the phenolic hydroxyl group present on PMCol, was less potent than PMCol at inhibiting androgenic responses. Therefore, the phenolic hydroxyl group of the chromanol ring contributes significantly to the antiandrogenic activity of PMCol. Other forms of vitamin E, such as β-, γ-, and δ-tocopherol differ from α-tocopherol by the number and location of methyl group substitutions on the chromanol ring (7). We can only speculate that the antioxidant moieties of other forms of vitamin E also possess antiandrogenic activity with potencies that vary depending on the specific methyl group substitutions present on the chromanol ring.

A variety of dietary agents have been identified that have antiandrogenic activity in prostate carcinoma cells. However, the mechanism of antiandrogenic activity observed by dietary antiandrogens may vary. For example, curcumin, a component of turmeric, was reported to down-regulate AR protein levels in LNCaP cells, which effectively attenuates androgenic responses (27). In contrast, indole-3-carbinol, a component of cruciferous vegetables, when converted to diindolylmethane was reported to act as a potent inhibitor of androgen binding in LNCaP cells but does not affect AR protein levels (28). Zhang *et al.* (21) have reported that vitamin E succinate is inhibitory to androgenic responses in LNCaP cells through down-regulation of AR protein levels, similar to the action of curcumin. By contrast, in the current study, we found that the antioxidant moiety of vitamin E, PMCol, effectively blocks androgen binding to the AR without affecting AR protein levels, similar to the effects observed with indole-3-carbinol derivatives (28). Therefore, dietary antiandrogens may serve as an effective means of modulating androgenic pathways through a variety of mechanisms affecting AR activity.

It is unclear how accurately the biological activity of α-tocopherol is modeled by the PMCol antioxidant moiety alone. PMCol has largely been investigated for its antioxidant activity associated with being the antioxidant moiety of vitamin E. For example, the antioxidant potency of PMCol was shown to be similar to that of α-tocopherol *in vitro* (29). In general, α-tocopherol plasma levels range between 5 and 30 μM (30), well within the range of antiandrogenic activity observed by PMCol in the current study. Due to the high lipophilicity of vitamin E, it is difficult to assess its antiandrogenic activity by cell culture analysis. However, we can speculate that due to the presence of the highly lipophilic phytyl chain, the subcellular distribution of vitamin E would limit its direct interaction with the AR, which resides in more aqueous subcellular compartments such as the cytoplasm and nucleus. Vitamin E can be metabolized to derivatives with greater water solubility, such as α-carboxyethylhydroxychroman (7, 31), which are structurally similar to PMCol and may have greater water solubility and a distinct cellular bioavailability compared with vitamin E. Thus, we hypothesize that metabolites of vitamin E may contact the AR *in vivo* and have antiandrogenic activity, analogous to that produced by PMCol in human prostate carcinoma cells.

In summary, the antioxidant moiety of α-tocopherol, PMCol, was found to inhibit androgen activity, likely through

competition for androgen binding to the AR, with resultant inhibition of androgen-sensitive biological pathways. PMCol was not found to possess androgen agonist or partial agonist activity and hence functions as a pure antagonist of androgen activity in the LNCaP and LAPC4 prostate carcinoma cell lines. Based on the results of the current study, PMCol may serve as a useful agent for modulating androgen activity *in vivo*. Importantly, the antiandrogenic activity of PMCol poses the possibility that the prostate cancer-preventive activity of vitamin E may be due, in part, to antiandrogenic effects of vitamin E or metabolites of vitamin E in the prostate. Currently, over 30,000 men die from prostate cancer each year in the United States (4). The prevention of prostate cancer through the action of dietary antiandrogens, such as vitamin E or its derivatives, may offer one means of reducing the devastation produced by this disease.

Acknowledgments

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